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Identification of Stem Cell Transcriptional Programs Normally Expressed in Embryonic and Neural Stem Cells in Alloreactive CD8$^+$ T Cells Mediating Graft-versus-Host Disease

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A hallmark of graft-versus-host-disease (GVHD), a life-threatening complication after allogeneic hematopoietic stem cell transplantation, is the cytopathic injury of host tissues mediated by persistent alloreactive effector T cells (T_E). However, the mechanisms that regulate the persistence of alloreactive T_E during GVHD remain largely unknown. Using mouse GVHD models, we demonstrate that alloreactive CD8$^+$ T_E rapidly diminished in vivo when adoptively transferred into irradiated secondary congenic recipient mice. In contrast, although alloreactive CD8$^+$ T_E underwent massive apoptosis upon chronic exposure to alloantigens, they proliferated in vivo in secondary allogeneic recipients, persisted, and caused severe GVHD. Thus, the continuous proliferation of alloreactive CD8$^+$ T_E, which is mediated by alloantigenic stimuli rather than homeostatic factors, is critical to maintaining their persistence. Gene expression profile analysis revealed that although alloreactive CD8$^+$ T_E increased the expression of genes associated with cell death, they activated a group of stem cell genes normally expressed in embryonic and neural stem cells. Most of these stem cell genes are associated with cell cycle regulation, DNA replication, chromatin modification, and transcription. One of these genes, Ezh2, which encodes a chromatin modifying enzyme, was abundantly expressed in CD8$^+$ T_E. Silencing Ezh2 significantly reduced the proliferation of alloantigen-activated CD8$^+$ T cells. Thus, these findings identify that a group of stem cell genes could play important roles in sustaining terminally differentiated alloreactive CD8$^+$ T_E and may be therapeutic targets for controlling GVHD.

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INTRODUCTION

Upon antigen-presenting cell (APC) activation, T cells are "programmed" to undergo clonal expansion, generating large numbers of effector T cells (T_E) while contracting to minimize their potentially lethal activity [1-6]. Consequently, the majority of CD8$^+$ T_E (~95%) may die after clearance of the antigen, with some memory T cells surviving contraction [4,6-8]. However, chronically activated T_E can be continually generated during chronic inflammatory conditions, such as responses to chronic infections, autoantigens, and alloantigens. A unique clinical example is graft-versus-host disease (GVHD), a life-threatening complication after allogeneic hematopoietic stem cell transplantation (HSCT) [9-13]. A hallmark of GVHD is the cytopathic injury mediated by persistent alloreactive T_E, which can occur within weeks and persist for years after transplantation [10-15]. GVHD therapy, which typically targets T_E, has disappointing response rates (~40%) [16]. However, the molecular mechanisms that regulate the persistence of alloreactive T cells during GVHD remain largely unknown.

Emerging evidence indicates that a group of stem cell signals may play important roles in antigen-experienced memory T cells. CD8$^+$ memory T cells have the ability to self-renew to survive the lifetime of an individual and can rapidly generate protective T_E upon antigenic rechallenge [1-5]. Gene expression

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profile analysis reveals that $CD8^+$ memory T cells and long-term hematopoietic stem cells (HSCs) share a self-renewal transcriptional program [17]. Furthermore, antigen-stimulated $CD8^+$ T cells undergo an asymmetrical division to regulate the generation of long-term memory T cells [18]. Thus, memory T cells are considered to be stem cell-like cells [1,3,4,19]. Interestingly, Wnt/β-catenin signaling, which is essential for proliferation and self-renewal of adult stem cells [20], has been shown to regulate the generation of $CD44^{lo}CD62L^{hi}CD122^{hi}Bcl\text{-}2^{hi}Sca\text{-}1^{hi}$ $CD8^+$ T memory stem cells (T_{MSC}) [21]. These $CD8^+$ T_{MSC} have greater ability than either $CD44^{hi}CD62L^{hi}$ central memory (T_{CM}) or $CD44^{hi}CD62L^{lo}$ effector memory T cells (T_{EM}) to proliferate and generate T_E, thereby destroying tumors [21]. This supports our previous observation that $CD8^+$ T_{MSC} are important for sustaining alloreactive T_E mediating GVHD [15]. However, these data do not explain why alloreactive $CD8^+$ T_E can persist and cause severe GVHD in secondary recipients [14,15]. Given that T_E and memory T cells are developmentally linked to each other [1-6,22], we asked whether alloreactive T_E exposure to chronic alloantigens proliferate and persist through reactivation of distinct families of stem cell genes.

Using mouse models of human GVHD directed against minor histocompatibility antigens (miHAs), we demonstrate that alloantigenic stimuli rather than homeostatic factors are critical to sustaining continuous proliferation of alloreactive $CD8^+$ T_E to counteract their massive apoptotic death. We found that a group of stem cell genes normally expressed in embryonic stem cells (ESCs) and neural stem cells (NSCs) was activated in these proliferating alloreactive $CD8^+$ T_E upon chronic exposure to alloantigens. Most of these stem cell genes are associated with DNA replication, cell cycle regulation, chromatin modification, and transcription. Silencing 1 of these genes, Ezh2, which encodes an enzyme with methyltransferase activity, inhibited the proliferation of alloantigen-activated T cells. Thus, these stem cell genes could be important therapeutic targets for modulating allogeneic T cell responses and GVHD.

MATERIALS AND METHODS

Mice

We purchased C57BL/6 (B6; H-$2D^b$, $CD45.2^+$), B6.SJL-*Ptprc*^{a} (B6/SJL, H-$2D^b$, $CD45.1^+$), C3H.SW (H-$2D^b$, $CD45.2^+$, and $Ly9.1^+$) mice, BALB/b (H-$2D^b$, $CD45.2^+$), B6.β_2 microglobulin gene-deficient mice (B6.$B_2M^{-/-}$), and BALB/c (H-$2D^d$, $CD45.2^+$) from Jackson Laboratory (Bar Harbor, ME, USA). We supplied transplant recipients with drinking water containing neomycin sulfate and polymyxin B (Sigma, St. Louis, MO, USA) as previously described [23]. The Institutional Animal Care and Use Committee of the University of Michigan approved all mouse protocols.

Antibodies, Cell Lines, Cytokines and Flow Cytometry Analysis

All antibodies (Abs) used for immunofluorescence staining were obtained from BD Bioscience Pharmingen (San Diego, CA, USA). Microbead-conjugated Abs and streptavidin were purchased from Miltenyi-Biotech (Auburn, CA, USA), and all recombinant cytokines including IL-2, IL-4, IL-15, granulocyte-monocyte colony-stimulating factor (GM-CSF), stem cell factor (SCF), and tumor necrosis factor-α (TNF-α) were from R&D Systems (Minnerapolis, MN, USA). miHA peptide H60/MHC-I dimmers were prepared by conjugating H60 peptide to MHC-I dimmers as instructed by the manufacturers (BD Bioscience). We performed immunofluorescence analyses of cell surface phenotypes and intracellular cytokines using FACScan and Canto cytometer (Becton Dickinson, San Jose, CA, USA) as previously described [23].

For 5-bromo-2′-deoxyuridine (BrdU) incorporation experiments, mice were given sterile drinking water containing 0.8 mg/mL BrdU (Sigma) for 3 days. BrdU labeling was performed as previously described [24]. In brief, after surface staining, cells were resuspended in cold 0.15 NaCl, fixed by addition of cold 95% ethanol, incubated for 30 minutes on ice, and washed with phosphate-buffered saline (PBS). The cells were then fixed using fixation solution from BD Cytofix/Cytoperm™ Kit (BD Bioscience) for 30 minutes, pelleted, and then incubated at 37 °C for 30 minutes with 50 KU of DNase I (Sigma) in 0.15 NaCl and 4.2 mM $MgCl_2$, pH5. After washing, cells were stained with FITC-conjugated anti-BrdU for 60 minutes at room temperature, followed by flow cytometry analysis.

Cell Preparations

T cell-depleted bone marrow (TCD BM) was prepared by incubating donor BM with microbead-conjugated anti-CD4 Ab and anti-CD8 Ab as previously described [23]. $CD8^+$ T cells were magnetically isolated from spleens and lymph nodes of mice using microbead-conjugated anti-CD8 Ab (MiniMACS, Miltenyi Biotech). $CD8^+$ T cell subsets were further separated using fluorescence activated cell sorter (MoFlo, Beckman Coulter, Fullerton, CA, USA). The purity of each sorted T cell subset was consistently more than 92%. Donor $CD8^+$ T cells were labeled with fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) as described [23]. We prepared mature DCs from B6 BM as described [25].

GVHD Induction

Mice underwent allogeneic BMT as previously described [23]. Briefly, for the C3H.SW anti-B6 mouse

GVHD model, we irradiated B6/SJL recipients using a split-dose totaling 10.0 Gy from a ^{137}Cs source. We mixed donor C3H.SW TCD BM (5×10^6) with or without C3H.SW CD44^{lo}CD62L^{hi} CD8$^+$ naïve T cells (T_N) (2.0×10^6) and transplanted into lethally irradiated B6/SJL recipients (4 to 8 mice per group per experiment). In some experiments, donor C3H.SW CD44^{lo}CD62L^{hi} CD8$^+$$T_N$ (2.0×10^6) were transplanted together with B6/SJL TCD BM (0.5×10^6) into lethally irradiated B6/SJL recipients. In the B6/SJL anti-BALB/b mouse GVHD model, we mixed B6 TCD BM (5×10^6) with or without CFSE-labeled B6/SJL CD44^{lo}CD62L^{hi} CD4$^+$ and CD8$^+$ T_N (2.5×10^6 T cells for each) and transplanted into lethally irradiated BALB/b recipients.

Array-Based mRNA Assays

In 3 repeated experiments, donor alloreactive day 14 CD8$^+$ T_{MSC} and T_E were highly purified, respectively, from 4 B6/SJL mice receiving donor CD44^{lo}CD62L^{hi} CD8$^+$ T_N derived from 6 C3H.SW mice. Donor CD44^{lo}CD62L^{hi} CD8$^+$ T_N were highly purified from pooled CD8$^+$ T cells of 2 C3H.SW mice in 3 separate experiments. Total RNA was prepared from these T cell subsets using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). Biotinylated cDNA was prepared for each sample from 600 ng total RNA using 2 rounds of reverse-transcription and T7 promoter-based in vitro transcription following hybridization to the arrays. Hybridization, scanning, and image analysis of the arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Mouse Genome 430A 2.0 Arrays containing 22,690 probe sets (Affymetrix) were used to broadly compare the transcription profile of CD8$^+$ T_E and T_{MSC} to that of T_N. The array data are available from Gene Expression Omnibus, accession GSE13743. Using publicly available software [26], we computed trimmed averages of PM-MM differences for each probe set and quantile-normalized these after scaling the arrays to give average probe set values of 1500 units. We then log-transformed using log (max[x+50;0] + 50). Using a 1-way analysis of variance (ANOVA) model we selected transcripts that gave $P < .01$ for comparing pairs of groups that also gave at least a 1.5-fold difference from the means for the paired groups, computed based on differences in the means of log-transformed data. We collapsed probe sets to 13,142 distinct genes using Entrez gene IDs. Data can be viewed from the following Web site: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=tjunpugmcoqomxc&acc=GSE13743.

Western Blot

Samples were separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Millipore Corporation, Bedford, MA, USA). After an overnight incubation at 4 °C with primary Abs, membranes were washed 5 times and probed with HRP-conjugated secondary Ab (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature, and immunoreactivity was detected by chemiluminescence (GE Healthcare, Piscataway, NJ).

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from the sorted CD8$^+$ T cell subsets using TRIzol (Invitrogen Life Technologies). Real-time RT-PCR was performed using a SYBR green PCR mix (ABI Biosystem, Foster City, CA) in the Realplex^2 Eppendorf Real-time PCR instrument (Eppendorf AG, Westbury, NY, USA). Gene expression levels were calculated relative to the 18S gene. The primer sequences used for real-time RT-PCR include: 18S (5′-GCTGCTGGCACCAGACTT and 3′-CGGCTACCACATCCAAGG), Ifng (5′-ATGAACGCTACACACTGCATC and 3′-CCATCCTTTTGCCAGTTCCTC), Granzyme B (5′-CCACTCTCGACCCTACATGG and 3′-GGCCCCCAAAGTGACATTTATT), Ezh2 (5′-TGCCTCCTGAATGTACTCCAA and 3′-AGGGATGTAGGAAGCAGTCATAC), Tacc3(5′-GAGATGGGGAAGTCCGTTGATG and 3′-CTCTGCTTGGGCCTTGCTGTGT), Birc5 (5′-AACTACCGCATCGCCACCTTC and 3′-TTCTTCCATCTGCTTCTTGACA), Hells (5′-GGGGAGTACCTGGACCTTTTCTTG and 3′- CTGCAGTGTCCCTTGTCTTTTGTG), Pd1(5′- ACCCTGGTCATTCACTTGGG, and 3′-CATTTGCTCCCTCTGACACTG), p18 (5′- GTAAACGTCAACGCTCAAAATGG and 3′-GAACCTGGCCAAGTCGAAGG), Casp4 (5′-ACAAACACCCTGACAAACCAC and 3′-CACTGCGTTCAGCATTGTTAAA), and Bcl2 (5′-GTCGCTACCGTCGTGACTTC and 3′-CAGACATGCACCTACCCAGC).

Lentiviral Vector Construction and Viral Production

Doxyclycline (Dox) regulated lentiviral vector pLVPT-rtTRKRAB2SM2 (pLVPT^{off}) was obtained from Addgene (Cambridge, MA) [27]. We cloned short-hairpin RNA duplex that specifically targets Ezh2 (Ezh2-shRNA, 5′CGCGTCCCC*AAGAGGTTCAGAAGAGCTG*TTCAAGAGA*CAGCTCTTCTGAACCTCTT*TTTTTGGAAAT3′) [28] into this pLVPT^{off}, in which Ezh2-shRNA and GFP are separately driven by H1 promoter and phosphoglycerate kinase (PGK) promoter (named Ezh2-shRNA/GFP-pLVPT^{off}), as previously described [27]. Lentiviral vector encoding scrambled shRNA and GFP was generated as control (named Con-shRNA/GFP-pLVPT^{off}). In the absence of Dox, Ezh2-shRNA and

GFP will be induced, whereas addition of Dox will repress the expression the transcription of both shEzh2 and GFP [27]. Production of lentiviruses was done in 293T cells as described [27].

In Vivo Reconstitution of T Cells with Inducible Knockdown of Ezh2

C-kit$^+$ hematopoietic cells were magnetically isolated from B6 mice and infected with Ezh2-shRNA-pLVPT^{off} in vitro as previously described [29], followed by transplantation into lethally irradiated B6 mice. To repress the expression of Ezh2-shRNA in HSCs during their hematopoietic and thymic reconstitution, all of these recipient mice were given sterile water containing Dox (2 mg/mL) from day −2 to 12 weeks after transplantation. HSCs infected with Control-shRNA-pLVPT^{off} were transplanted as control. Twelve weeks after transplantation, Dox was removed from these mice to induce the expression of Ezh2-shRNA. Seven days later, CD8$^+$ T cells were isolated from the spleens and lymph nodes of these mice. GFP$^+$CD8$^+$ T cells expressing Ezh2-shRNA (named Ezh2-shRNA GFP$^+$CD8$^+$ T cells), or Control shRNA (named Control-shRNA GFP$^+$CD8$^+$ T cells) were sorted using the BD FACSAria II cell sorter (BD Bioscience).

Ex Vivo Stimulation of CD8$^+$ T Cells

Sorted Ezh2-shRNA GFP$^+$CD8$^+$ T_N and control-shRNA GFP$^+$CD8$^+$ T_N were stimulated with anti-CD3 Ab and anti-CD28 Ab (2.5 μg/mL for each) in 96-well plate as previously described [14,15,30]. In some experiments, unfractionated splenic mononuclear cells that contained Ezh2-shRNA GFP$^+$CD8$^+$ T cells were cultured in the presence of allogeneic DCs or IL-7. The recovery number of GFP$^+$CD8$^+$ T cells was assessed by flow cytometry analysis.

Statistical Analysis

Survival in different groups was compared by using the log-rank analysis. Comparison of 2 means was analyzed using the 2-tailed unpaired Student *t*-test. Statistical analysis from the gene array is indicated in the text.

RESULTS

Alloantigen-Induced Continuous Proliferation Is Essential to Maintaining Chronically Activated Alloreactive CD8$^+$ T_E

Because CD8$^+$ T_E are known to be "terminally differentiated" and short-lived cells [1-6], we first determined under what conditions alloreactive CD8$^+$ T_E were able to persist in vivo and cause GVHD. We transplanted CFSE-labeled donor CD8$^+$ T_N from normal C3H.SW mice together with B6/SJL TCD BM into lethally irradiated B6/SJL recipients. This allowed us to strictly track the fate of infused donor mature T cells while inducing GVHD [15]. By day 14 after transplantation, donor alloreactive CD8$^+$ T_E became the dominant population (>80%) (named day 14 CD8$^+$ T_E, Figure 1A). They had undergone extensive division and produced significantly higher levels of IFN-γ and Granzyme B (Gzmb) (Figure 1A and B). When later adoptively transferred into lethally irradiated secondary allogeneic B6/SJL mice, all the day 14 CD8$^+$ T_E had extensively divided in vivo, expressed high levels of proliferating antigen Ki67 7 days after transplantation (Figure 1C). BrdU incorporation analysis showed that about 22% of day 14 CD8$^+$ T_E derived from primary GVHD recipient mice and 76% of alloreactive CD8$^+$ T_E recovered from secondary recipients of day 14 CD8$^+$ T_E had incorporated BrdU (Figure 1D). These results suggest that a substantial proportion of day 14 CD8$^+$ T_E are dividing during GVH reaction.

Interestingly, 4-fold fewer donor T cells were recovered from secondary recipients of day-14 CD8$^+$ T_E than that of donor CD8$^+$ T_N (Figure 1E). This was associated with significantly increased apoptotic death of donor T cells in day 14 CD8$^+$ T_E recipients compared to CD8$^+$ T_N recipients (Figure 1F). Furthermore, adoptive transfer of these day 14 CD8$^+$ T_E caused GVHD in secondary recipients, with 70% of them dying from the disease by day 75 after transplantation (Figure 1G). Thus, upon chronic exposure to alloantigens, alloreactive CD8$^+$ T_E continuously proliferated to persist while undergoing increased apoptotic death. However, all alloreactive CD8$^+$ T_E diminished in vivo without causing GVHD when adoptively transferred into secondary B6.$B_2M^{-/-}$ mice (data not shown). These results suggest that allogeneic stimuli could be essential to sustaining alloreactive CD8$^+$ T_E.

In lethally irradiated allogeneic recipient mice, both alloantigens and lymphopenia-related homeostatic factors (eg, self-antigen peptides and cytokines IL7 and IL-15) [31] could be responsible for the proliferation of alloreactive CD8$^+$ T_E. To assess the possible impact of alloantigens versus homeostatic factors in regulating the persistence of alloreactive T_E, we used the CD4$^+$ T cell-dependent B6 anti-BALB/b GVHD model, in which miHA H60-specific (H60$^+$) CD8$^+$ T_E could be tracked using miHA peptide/MHC class-I dimmer staining (Figure 2A) [14,32,33]. We highly purified H60$^+$CD8$^+$ T_E at day 12 posttransplantation from GVHD BALB/b mice receiving donor B6/SJL T_N (CD45.1), labeled with CFSE, and adoptively transferred together with B6/SJL CD4$^+$ T_E and donor B6 TCD BM into lethally irradiated congenic B6 (CD45.2$^+$) mice and allogeneic BALB/b mice. Lethal irradiation of congenic B6 mice creates a lymphopenic environment that might induce homeostatic proliferation and survival of adoptively

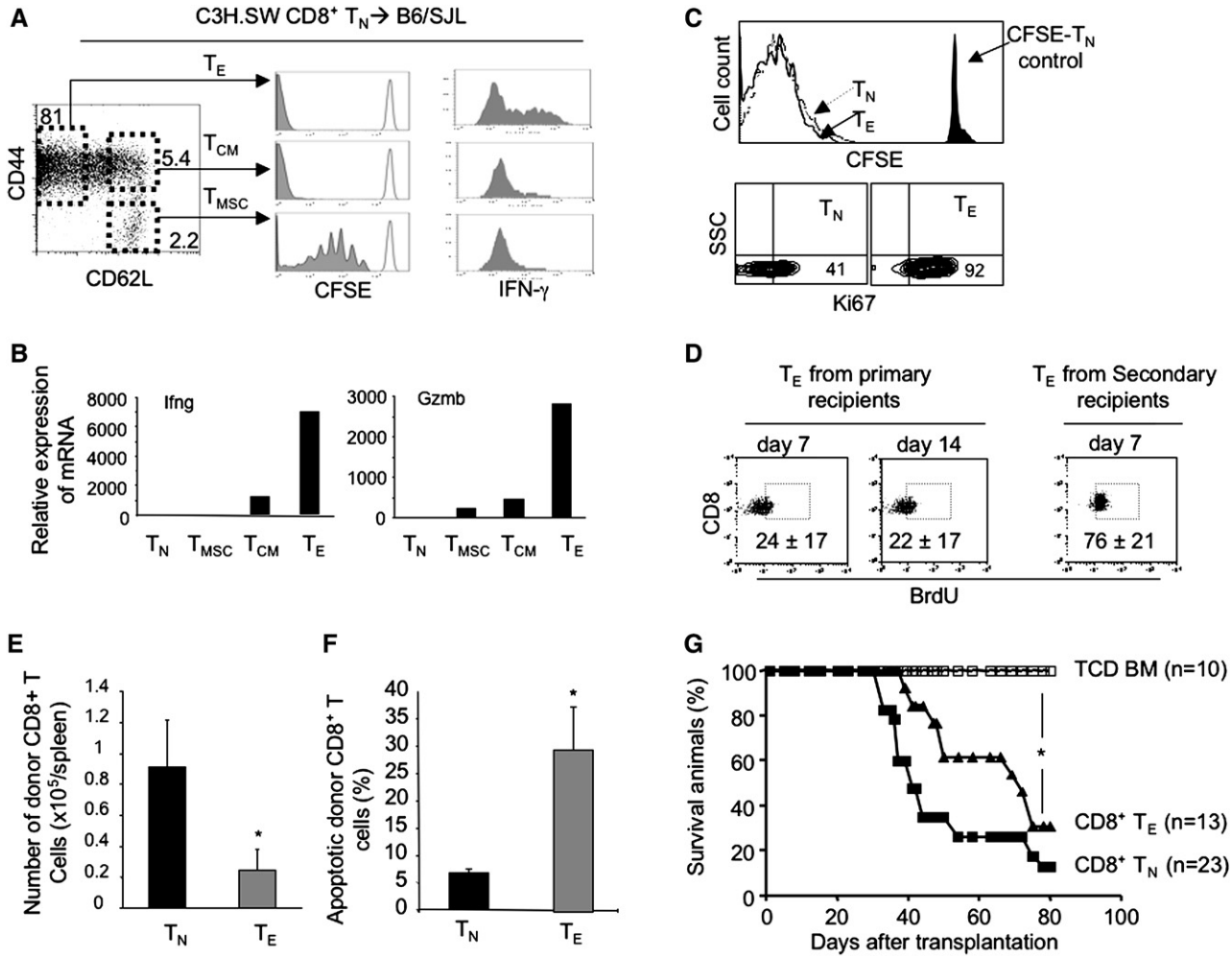


Figure 1. Alloreactive $CD8^+$ T_E are highly replicating cells causing GVHD. (A) CFSE-labeled donor $CD8^+$ T_N (2×10^6) derived from normal C3H.SW mice were transplanted with B6/SJL TCD BM (0.5×10^6) into lethally irradiated B6/SJL mice. Donor $CD8^+$ T cells were recovered at day 14 after transplantation from the spleen and lymph node of 3 recipients, enumerated, and stained for flow cytometric analysis. Histograms show the cell division based on CFSE dilution and production of IFN-γ by $CD8^+$ T_{MSC}, T_{CM}, and T_E. Data are representative of 3 independent experiments. (B) Real-time RT-PCR analysis shows the relative mRNA expression of selected genes in donor day 14 $CD8^+$ T_E, T_{CM} and T_{MSC}. Data are representative of 3 independent experiments. (C, D) Day 14 $CD8^+$ T_E were relabeled with CFSE and adoptively transferred into lethally irradiated secondary B6/SJL mice. CFSE-labeled C3H.SW $CD8^+$ T_N were transferred as controls. Donor T cells were recovered at day 7 after adoptive transfer, enumerated, and analyzed for flow cytometry. Histogram shows the cell division of indicated cells. Nonstimulated CFSE-labeled $CD8^+$ T_N were used as nondividing cell control. Contour plots show the expression of Ki67 (C). For BrdU labeling, secondary recipient mice were given drinking water containing BrdU for 3 days prior to the end of the experiments. At day 7, donor cells were recovered, stained with BrdU, and analyzed by flow cytometry. Dot plots show the labeling of BrdU in gated $CD8^+$ T_E population (mean ± SD) (D). (E, F) As described in C, the number of donor T cells recovered from these secondary recipients ($n = 3$ for each group) was calculated (E) and the percentage of apoptotic cell was shown (F). Data are representative of 2 independent experiments. (G) Donor C3H.SW TCD BM (5×10^6) were transplanted alone, or with donor day 14 $CD8^+$ T_E (0.5×10^6) and donor $CD8^+$ T_N (1.0×10^6) into lethally irradiated secondary B6/SJL mice. Survival of animals was analyzed with the Kaplan-Meier method. *$P < .05$, significant difference.

transferred day 12 B6/SJL $CD8^+$ T cells in the absence of miHA H60, whereas lethally irradiated allogeneic BALB/b mice could provide persistent alloantigen-stimulation in addition to homeostatic factors.

We found that donor $H60^+CD8^+$ T_E were readily detected in the peripheral blood of secondary allogeneic BALB/b recipients (43.3 ± 19%) by day 14 after transplantation, but not in that of secondary congenic B6 recipients (0.1 ± 0.3%) (Figure 2B and C). Forty-three days after transfer, allogeneic BALB/b recipients showed about 80-fold more proliferating $H60^+CD8^+$ T cells than B6 congenic recipients (Figure 2D). These donor $H60^+CD8^+$ T_E from secondary allogeneic BALB/b recipients expressed high levels of CD69, suggesting a recent antigenic stimulation (Figure 2E). Furthermore, they produced high levels of IFN-γ and Granzyme B (Figure 2E) and caused GVHD in these secondary allogeneic BALB/b mice (Figure 2F). In separate experiments, we observed that adoptive transfer of donor alloreactive $CD8^+$ T_E alone also caused GVHD in secondary allogeneic BALB/b mice (Figure 2F), suggesting that CD4-help is not essential to already differentiated $CD8^+$ T_E to mediate GVHD. None of

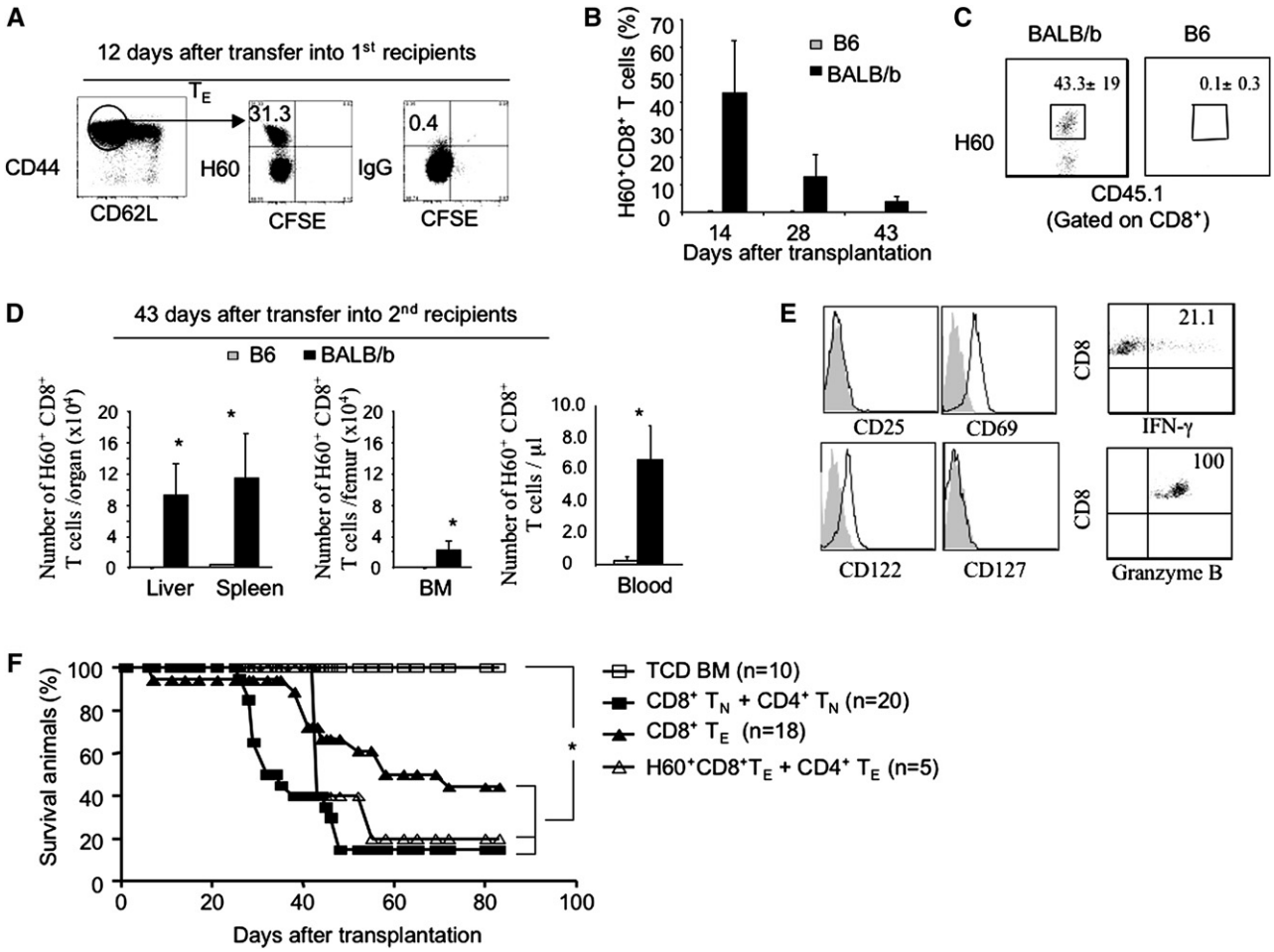


Figure 2. Proliferation and persistence of alloantigen-specific $CD8^+$ T_E depend on the presence of chronic alloantigen. (A) CFSE-labeled donor T cells ($CD4^+$ and $CD8^+$ T cells, 3×10^6 for each) from normal B6/SJL mice (CD45.1) were transplanted, together with B6 TCD BM (CD45.2, 5×10^6), into lethally irradiated (10 Gy) primary BALB/b recipients (CD45.2). Dot plots show percent of host miHA H60-specific $CD8^+$ T_E that were recovered at day 12 after transplantation from spleens and livers of primary GVHD BALB/b recipients (n = 4) of B6/SJLTCD BM and B6/SJL T cells. Data are representative of 3 independent experiments. (B, C, D) Donor alloreactive B6/SJL $H60^+CD8^+$ T_E (5×10^4) and $CD4^+$ T cells (3×10^5) were isolated 12 days after transplantation, respectively, from these primary GVHD BALB/b recipients (CD45.2) of B6/JL T cells, relabeled with CFSE, and adoptively transferred together with B6 TCD BM (5×10^6) into lethally irradiated secondary BALB/b recipients and congenic B6 recipients (CD45.2), respectively. The percentage of donor alloreactive $H60^+CD8^+$ T_E in the peripheral blood of these secondary recipients at day 14, 28, and 43 after adoptive transfer (B). Dot plots show the fraction of $H60^+CD8^+$ T_E in the peripheral blood (C). Donor alloreactive $H60^+CD8^+$ T_E recovered at day 43 from secondary recipients were calculated (D). Data are presented as mean ± SD. *$P < .01$. (E) Histograms show the surface markers of donor $H60^+CD8^+$ T_E isolated from the secondary allogeneic BALB/b mice (left), and dot plots show the production of IFN-γ and GZMB (right). Data shown in B, C, D, and E are representative of 2 independent experiments. (F) Survival rate of animals was analyzed by the Kaplan-Meier Method. *$P < .05$, significant difference.

these congenic B6 mice receiving donor alloreactive $CD8^+$ T_E developed clinical signs of GVHD (data not shown). Thus, alloantigenic stimuli rather than homeostatic factors are critical to the continual proliferation and persistence of alloreactive $CD8^+$ T_E during GVH reaction.

Gene Expression Profiles of Alloreactive $CD8^+$ T_E

To understand the molecular mechanisms by which alloreactive $CD8^+$ T_E acquire the ability to continually proliferate upon chronic exposure to alloantigens, we used Affymetrix Mouse Genome A430A 2.0 Array to broadly compare the gene expression profiles of day 14 $CD8^+$ T_E to that of $CD8^+$ T_N and $CD8^+$ T_{MSC} (Figure 3A). Compared to T_N, a total of 2744 distinct genes were upregulated (1359) or downregulated (1385) in $CD8^+$ T_E by a 1.5-fold that gave a $P < .01$ value for comparing pairs of groups. As predicted by our experimental data [14,15] and others [12,34], relative to $CD8^+$ T_N and T_{MSC}, $CD8^+$ T_E expressed significantly higher levels of genes known to be important for effector functions, including effector molecules, chemokines, and chemokine receptors (Table 1). As expected, many other genes engaged in T cell receptor (TCR) signaling pathway, glycolysis, MAPK pathway, and cell adhesion were also altered in $CD8^+$ T_E (Table 1).

To better define a specific gene signature of alloreactive T cells, we further tested each list of distinct genes for overrepresentation in 507 lists of Gene

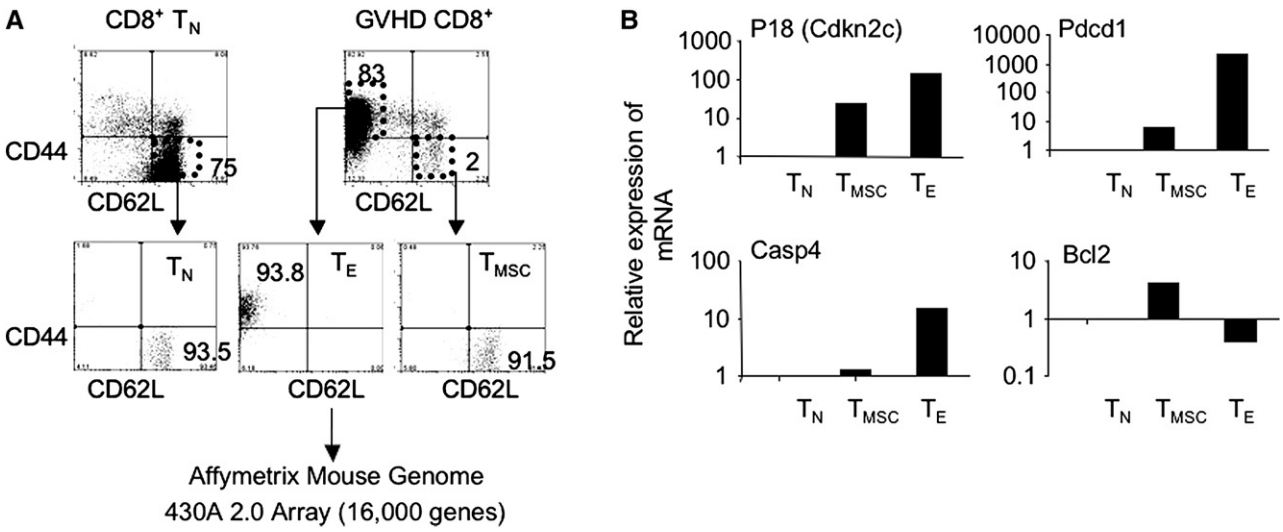


Figure 3. Array based mRNA arrays of alloreactive $CD8^+$ T cells. (A) Donor $CD44^{lo}CD62L^{hi}$ $CD8^+$ T_N (2×10^6) from C3H.SW mice (CD45.2) were transplanted with B6/SJL TCD BM (0.5×10^6) into lethally irradiated B6/SJL mice (CD45.1). Donor $CD8^+$ T cells were recovered at day 14 after transplantation from the spleens and lymph nodes of these recipients, magnetically purified, and stained with indicated Abs for flow cytometry cell sorting. Affymetrix Mouse Genome 430A 2.0 array was used to broadly compare the transcription profiles of these $CD8^+$ T_{MSC} and T_E to that of T_N cells. (B) Real-time RT-PCT analysis shows the relative expression of mRNA in each T cell subset. Data are representative of 3 independent preparations of alloreactive T cells.

Ontology (GO) terms for which there were at least 10 genes present on the array (Affymetrix Web site), as well as for 190 lists of pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/). Compared to both $CD8^+$ T_N and T_{MSC}, $CD8^+$ T_E demonstrated a signature expression of genes related to cell cycle, mitosis, apoptosis, and transcription and translation (Table 2).

Detailed analysis revealed that alloreactive $CD8^+$ T_E showed decreased expression of antiapoptotic gene Bcl2, but had increased genes related to apoptosis, including Pdcd1 (also named Pd1), Klrg1, Casp1, Casp3, Caps4, Casp7, Bax, and Bad (Table 1). In contrast, $CD8^+$ T_{MSC} expressed higher levels of Bcl2 than both T_N and T_E, whereas it only minimally changed in the expression of other pro-apoptotic genes (Table 1 and Figure 3B). When compared to $CD8^+$ T_N, $CD8^+$ T_E show significantly decreased expression of genes related to ribosome biogenesis and assembly, ribosome, translation, and transcription (Table 2). Real-time RT-PCR validated the expression some of these genes (Figure 3B).

Notably, alloreactive $CD8^+$ T_E also had 150-fold more expression of $p18^{Ink4c}$ (encoded by Cdkn2c) than $CD8^+$ T_N and 15-fold than $CD8^+$ T_{MSC} (Figure 3B). Previous studies have shown that $p18^{Ink4c}$ plays a critical role in negatively regulating cell proliferation and survival [35-38]. The loss of $p18^{Ink4c}$ in T cells causes their hyperproliferation response and proliferation disorder [38,39].

Altogether, this transcriptional signature confirmed that alloreactive $CD8^+$ T_E are replicating cells, but are more likely susceptible than $CD8^+$ T_{MSC} to apoptotic death and senescence, consistent with our previous observations [15].

Alloreactive $CD8^+$ T_E Activate Stem Cell Transcriptional Programs

Next we tested 1687 curated gene lists from Molecular Signature Database v2 (MSigDB v2) [40] to inquire which transcriptional program(s) could be associated with the continuous proliferation property of alloreactive $CD8^+$ T_E. Based on 1-sided Fisher's exact test, we identified that transcripts increased in $CD8^+$ T_E were significantly enriched in lists of genes increased in NSCs (345 of 1636 genes, $P = 7.3 \times 10^{-44}$) and ESCs (227 of 1193 genes, $P = 1.7 \times 10^{-21}$) (Figure 4A), which were identified by Ramalho-Santos et al. [41]. These alloreactive $CD8^+$ T_E-related ESC genes and NSC genes are listed in Table 3 and Table 4. Among them, 171 genes were shared by ESCs and NSCs, 56 appeared in ESCs, and 174 were found only in NSCs. Thus, 401 of 1369 (29%) of transcripts that were increased in alloreactive $CD8^+$ T_E were enriched for ESCs and/or NSCs (Figure 4A). In contrast, transcripts decreased in $CD8^+$ T_E were overrepresented among HSC-related genes (253 of 1279, 19.8%, $P = 2.2 \times 10^{-25}$) (Figure 4A).

Like T_E, the 543 transcripts increased in T_{MSC} versus T_N showed significant overrepresentation of ESC- and NSC-related genes ($P = 4.8 \times 10^{-5}$ and 3.4×10^{-6}, respectively) (Figure 4A). Notably, 72% of ESC- and NSC-related genes selected as increased in T_{MSC} were also increased in T_E (87 of 121 genes; Tables 3

Table 1. Altered Transcripts in T_{MSC} and T_E Compared to T_N

		Fold Change				Fold Change				Fold Change	
Gene Symbols	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbols	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbols	Gene ID#	T_{MSC}/T_N	T_E/T_N
Effector function				**Glycolysis/Gluconeogenesis**				**mTOR pathway**			
Gzmb	14939	38.3	306.4	Hk2	15277	2	7.1	Rps6kb2	58988		2.7
Ifng	15978	4.2	211	Tpi1	21991	1.9	4.1	Akt1	11651		2.1
Fasl	14103		66.3	Eno3	13808		4	Eif4e2	26987		2
Klrk1	27007	2.9	3.3	Gapdh	14433	1.5	3.8	Rps6ka1	20111		1.7
Prf1	18646		3	Acyp2	75572	1.8	2.4	Eif4b	75705		−1.9
Tnfsf10	22035		2.7	Pfkp	56421	1.5	1.9	Tsc1	64930		−2
Klra3	16634		2.3	Bpgm	12183		1.6	Rps6ka2	20112		−2.9
Chemokine-chemokine receptor interactions				**MAPK signaling pathway**				**Adhesion and transendothelial migration**			
Ccl5	20304	15.9	453.5	Stmn1	16765	5	19.3	Cd44	12505		14.2
Ccl4	20303		69.6	Dusp1	19252	1.8	4.9	Hmmr	15366		7.2
Ccl3	20302		42	Map3k8	26410	7.6	3.4	Itgb1	16412		5.6
Ccr5	12774	4.7	32.8	Gadd45b	17873		3.2	Spp1	20750		4.5
Ccr2	12772		10.8	Gadd45g	23882	15.3	2.7	Itga4	16401		2.7
Cxcr3	12766	4.1	8.3	Dusp3	72349	1.8	2.5	Sdc4	20971		2.5
Cx3cr1	13051		4.8	Mapkapk2	17164		1.8	Itgav	16410		2.4
Xcl1	16963		4.7	Mapk3	26417		1.8	Lamc1	226519		2
Cxcl10	15945		3.1	Map2k3	26397		1.7	Itga3	16400		2
Cxcl10	15945		3.1	Mapk8	26419	−1.6		Npnt	114249		1.9
Ccr6	12458		2.9	Mapk14	26416	−1.6		Itgb2	16414		1.9
Ccl6	20305		2.7	Map2k6	26399	−1.6		Vcl	22330		1.7
Ccr7	12775		−29.1	Dusp2	13537	−1.6		Tnc	21923		1.6
Cytokine-cytokine receptor interactions				**Jak-STAT signaling pathway**				Pxn	19303		1.5
Il10ra	16154	1.7	9.9	Pim1	18712	2.3	2.3	**Apoptosis**			
Csf1	12977	−2	6.4	Jak3	16453	1.8	1.9	Casp3	12367		5.4
Il2rb	16185	1.7	6.3	Socs5	56468	−1.7	−1.7	Capn2	12334		2.3
Il10	16153		6.1	Stat6	20852		−1.7	Bid	12122		2.2
Csf2	12981		4.7	Stat1	20846	2	−1.8	Apaf1	11783		2.2
Il15ra	16169	2	2.5	Jak1	16451	−1.5	−1.8	Casp7	12369		2.1
Il12rb1	16161	1.9	2.5	Socs3	12702	2.6	−2.4	Bax	12028		1.8
Il4ra	16190		−2.8	Socs1	12703	1.9		Bcl2l1	12048		1.6
Il6st	16195	−1.6	−4.3	Myc	17869	1.5		Bad	12015		1.5
Il7r	16197	−1.9	−7.3	**Toll-like receptor signaling pathway**				Bcl2	12043	2	−1.5
Il6ra	16194		−18.6	Tlr7	170743	2.6		Trp53	22059	1.9	
Il10rb	16155	1.6		Tlr6	21899	1.5					
TCR signaling				**TCR signaling**				**TCR signaling**			
Tbx21	57765	8.8	21.5	Cdc42	12540		1.6	Ikbkb	16150		−2.5
Map3k8	26410	7.6	3.4	Hras1	15461	1.5	1.5	Pak1	18479		−5.8
Nfatc1	18018		2.6	Ikbkg	16151		−1.5	Pdk1	228026		−6.3
Fyn	14360		2.1	Itk	16428	−1.5	−2	Jun	16476	6.1	
Ppp3cc	19057	1.6	1.7	Map3k14	53859	−1.5	−2.2	Fos	14281	4	
Zap70	22637		1.7	Tec	21682		−2.5	Lcp2	16822	−1.6	

TCR indicates T cell receptor.

and 4). Thus, despite their different proliferation capability, both alloreactive $CD8^+$ T_E and T_{MSC} share some common stem cell transcriptional programs.

To validate our observations using an unsupervised approach, we obtained Sarkar's et al. dataset (GSE10239) [42], fit 1-way ANOVA models, and performed probe-set selections identical to those of our array data. In their experiments, both $KLRG1^{Int}$ and $KLRG1^{High}$ P14 $CD8^+$ T_E specific to lymphocytic choriomenigitis (LCMV)-gp33 showed probe set differences compared to T_N that had significantly overlaps with probe sets that we found to differ between alloreactive $CD8^+$ T_E and T_N in our GVHD model ($P < 10^{-200}$, by Fisher's Exact test in both cases). Most importantly, as with our alloreactive $CD8^+$ T_E, P14 $CD8^+$ T_E highly enriched for ESC- and NSC-related genes (Figure 4B). In contrast, P14 $CD8^+$ memory cells decreased the expression of ESC- and NSC-related genes, whereas it activated genes enriched for HSCs (Figure 4B). These data independently confirmed our gene microarray data.

Characterization of Stem Cell Genes Activated in Alloreactive $CD8^+$ T_E

These observations were surprising to us because $CD8^+$ T_E have been believed to be terminally differentiated cells [1,43]. To understand what these $CD8^+$ T_E-related stem cell genes were, we assigned these genes to several functional categories by filtering for keywords in the GO terms as previously described by Ramalho-Santos et al. [41]. As shown in Table 5, we found that $CD8^+$ T_E shared the similarity with ESCs and NSCs in the expression of genes associated with: (1) regulation of cell cycle, (2) resistance to stress, (3) chromatin modification and transcription/

Table 2. Most Significantly Enriched GO Terms and KEGG Pathways

A) Tests of 507 Gene Ontology (GO) Biological Process Terms with at Least 10 Genes Present on the Arrays

Comparison	Direction	Number of Probe Sets Selected (of 22,690)	Number of Distinct Genes Selected (of 13,142)	GO Term Title	Number of Genes with GO Term on the Array	Number of Those Genes Selected	*P*-Value	Average Number of GO Terms This Good in 100 Permutations
T_{MSC} versus T_N	up in T_{MSC}	672	543	cell cycle	335	41	4.1E-10	0
				immune response	263	29	1.4E-06	0
				DNA replication	96	16	1.8E-06	0
				mitosis	109	17	2.2E-06	0
				cell division	173	22	2.6E-06	0
				regulation of progression through cell cycle	185	20	8.1E-05	0.003
T_{MSC} versus T_N	up in T_N	545	448	transcription	1125	72	1.1E-07	0
				regulation of transcription, DNA-dependent	1486	86	4.6E-07	0
T_E versus T_{MSC}	up in T_E	1486	1098	cell cycle	335	102	1.5E-32	0
				mitosis	109	53	1.1E-28	0
				cell division	173	66	1.1E-27	0
				DNA replication	96	40	6.7E-19	0
				apoptosis	296	54	3.1E-08	0
				DNA replication initiation	13	9	1.0E-07	0
T_E versus T_{MSC}	up in T_{MSC}	1372	1036	ribosome biogenesis and assembly	47	21	9.6E-12	0
				translation	259	51	7.5E-10	0
T_E versus T_N	up in T_E	1817	1359	cell cycle	335	109	2.6E-29	0
				mitosis	109	52	2.9E-23	0
				cell division	173	67	3.5E-23	0
				DNA replication	96	41	1.8E-16	0
				DNA replication initiation	13	10	2.9E-08	0
				apoptosis	296	59	5.1E-07	0
				DNA recombination	35	13	2.4E-05	0
				regulation of progression through cell cycle	185	38	2.6E-05	0
T_E versus T_N	up in T_N	1837	1359	ribosome biogenesis and assembly	47	18	5.3E-07	0
				translation	259	49	3.3E-05	0
				transcription	1125	158	6.9E-05	0

B) Tests of 190 KEGG Pathways

Comparison	Direction	Number of Probe Sets Selected (of 22,690)	Number of Distinct Genes Selected (of 13,142)	Pathway Title	Number of Genes in Pathway on the Array	Number of Those Genes Selected	*P*-Value	Average Number of Pathways This Good in 100 Permutations
T_{MSC} versus T_N	up in T_{MSC}	672	543	Cell cycle	102	16	4.1E-06	0
				Pyrimidine metabolism	80	12	1.0E-04	0
				Jak-STAT signaling pathway	122	14	5.0E-04	0.02
T_{MSC} versus T_N	up in T_N	545	448	Wnt signaling pathway	133	11	5.8E-03	0.3

(*Continued*)

translation regulation, (4) cell survival and death, (5) signaling, and (6) others. In cell cycle category, $CD8^+$ T_E increased both negative (eg, $p18^{Ink4c}$ and $p21^{Cip1}$) and positive cell cycle regulators (eg, Ccna and Ccnb). Interestingly, after removing all these cell cycle genes from our array data and Ramalho-Santos's stem cell data set [41], we found that the rest of transcripts increased in alloreactive $CD8^+$ TE remained significant overrepresentation of ESC- and NSC-related genes ($P < 5.0^{-15}$, by Fisher's Exact test in both cases). Our further evaluation of Sarkar's et al. [42] and Ramalho-Santos's et al. [41] data sets without these cell cycle genes also revealed significant enrichments in P14 $CD8^+$ TE for ESC- and NSC-related transcripts ($P < 4.2^{-82}$ in both cases). Thus, cell cycle genes are not the only attributes to the similarity of gene expression between $CD8^+$ T_E and embryonic and neural stem cells.

Genes engaged in resistance to stress represented were a large group of stem cell transcripts activated in $CD8^+$ T_E. This group of genes included transcripts of DNA replication and repair, ubiquitin/proteasome, metabolism, and electron transport (Table 5). These genes are considered to be related to the stress condition of stem cells [41]. For example, genes including Coq7, Ube2l3, Nedd4, and Psma play important role in modifying abnormal or short-lived proteins [44,45]. DNA repair gene Rad51 is critical to maintaining chromosomal stability and preventing genetic mutation potentially occurring during cell division [41,46]. Genes associated with metabolisms, such as Gldc and Gsto1, are critical to amino acid and antioxidant metabolism [47,48]. The functions of these genes in T cells and stem cells remain largely unknown.

Another important finding was that $CD8^+$ T_E activated many genes engaged in DNA methylation, chromatin modification, transcription, and survival in ESCs and NSCs. For example, Uhrf1 protein forms complexes with DNA methyltransferase Dnmt1, which may result in an inheritable DNA methylation [49]. Hells (also known as Lsh) protein associates with Dnmt3a and Dnmt3b in embryonic cells for DNA methylation and transcription [50]. Tacc3 protein can activate gene transcription even prior to demethylation [51]. Birc5 (also known as Survivin), is an apoptosis inhibitor in both normal and malignant cells [52]. Ezh2, which encodes a chromatin modifying enzyme with methyltransferase activity, orchestrates gene expression in both embryonic and adult stem cells [53-55]. The representative gene expression of these chromatin modifiers and transcriptional regulators were shown in Figure 5A, and validated by real-time RT-PCR (Figure 5B). Thus, genes in this category have multiple roles in controlling cell fate, self-renewal, differentiation, survival, and memory function.

Finally, we noted that $CD8^+$ T_E did not activate those genes associated with pluripotency of ESCs,

Table 2. (*Continued*)

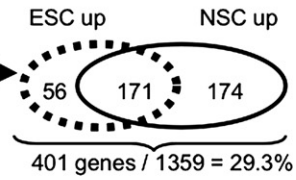
B) Tests of 190 KEGG Pathways

Comparison	Direction	Number of Probe Sets Selected (of 22,690)	Number of Distinct Genes Selected (of 13,142)	Pathway Title	Number of Genes in Pathway on the Array	Number of Those Genes Selected	*P*-Value	Average Number of Pathways This Good in 100 Permutations
T_E versus T_{MSC}	up in T_E	1486	1098	Cell cycle	102	39	6.5E-17	0
				Natural killer cell-mediated cytotoxicity	101	23	7.3E-06	0
T_E versus T_{MSC}	up in T_{MSC}	1372	1036	Ribosome	71	29	1.9E-14	0
T_E versus T_N	up in T_E	1817	1359	Cell cycle	102	45	1.3E-18	0
				Proteasome	30	13	3.2E-06	0
				Natural killer cell- mediated cytotoxicity	101	25	2.5E-05	0
T_E versus T_N	up in T_N	1837	1359	Ribosome	71	26	5.0E-09	0

T_{MSC} indicates T memory stem cells; T_N, naïve T cells; T_E, effector T cells; KEGG. Kyoto Encyclopedia of Genes and Genomes.

A **Activation of stem cell transcriptional programs in alloreactive CD8⁺ T_E**

Differential gene expression		Enriched transcripts in stem cells: ESC (1193)	NSC (1636)	HSC(1279)
Increased transcripts as compared to T_N				
T_{MSC}	543	77 (p=4.8E -5)	104 (p=3.4E -6)	60 (p=0.16)
T_E	1359	227 (p=1.7E -21)	345 (p=7.3E -44)	152 (p=0.03)
Shared between T_{MSC} and T_E	309	52 (p=8.9E -6)	77 (p=9.9E -10)	32 (p=0.382)
Decreased transcripts as compared to T_N				
T_{MSC}	448	38 (p=0.70)	61 (p=0.24)	82 (p=1.1E -8)
T_E	1385	133 (p=0.25)	158 (p=0.10)	253 (p=2.2E -25)
Shared between T_{MSC} and T_E	267	23 (p=0.64)	28 (p=0.51)	59 (p=1.1E -9)



B

Enrichment of stem cell genes in LCMV-gp33 specific CD8⁺ T_E and CD8⁺ memory T cells in the array data of Sarkar et al. (J Exp Med. 2008;205:625-640)

Differential gene expression		Enriched transcripts in stem cells: ESC (1166)	NSC (1604)	HSC(1255)
Increased transcripts as compared to T_N				
T_E-KLRG^{Hi}	1427	303 (4.1E -98)	393 (2.4E -121)	114 (2.0E -3)
T_E-KLRG^{Int}	1511	308 (5.2E -95)	400 (2.0E -117)	130 (5.5E -5)
Memory T cells	1342	96 (0.01)	151 (3.8E -6)	166 (1.2E -18)
Decreased transcripts as compared to T_N				
T_E-KLRG^{Hi}	1546	66 (1.0)	123 (0.46)	251 (6.4E -49)
T_E-KLRG^{Int}	1599	66 (1.0)	117 (0.82)	240 (1.3E -40)
Memory T cells	2212	346 (1.3E -74)	485 (5.8E -111)	237 (2.0E -18)

Figure 4. Acquisition of stem cell transcriptional programs in alloreactive CD8$^+$ T cells. (A) Genes distinctively expressed by CD8$^+$ T_{MSC} and T_E relative to T_N were analyzed for functional set enrichment analysis using curated gene lists from the Ramalho-Santos's array data. (B) Acquisition of stem cell transcriptional programs in P14 LCMV-gp33-specific CD8$^+$ T cells. Genes differentially expressed by CD8$^+$ T_E-KLRG1^{Hi}, CD8$^+$ T_E-KLRG1^{Hint} and CD8$^+$ memory T cells relative to T_N were analyzed for enrichment for the same stem cell gene lists. Sizes of stem cell gene lists differ slightly from that in Figure 4A because Sarkar's et al. study used Affymetrix Mouse_430_2, which also had slightly different probe-set annotation than our arrays.

such as Oct4, Sox2, Klf4, Nanog, and c-Myc [56] (Figure 5A; other data not shown). Furthermore, genes associated with HSC self-renewal were decreased in CD8$^+$ T_E. For example, CD8$^+$ T_E markedly downregulated the expression of many genes related to receptor, signaling, and transcription that are normally expressed in HSCs, such Il6ra, Il6st, Smad4, Smad7, and c-Myc (Table 6 and Figure 5A). Among them, Smad4 and Smad7 have been shown to be required for self-renewal and quiescence of HSCs [57].

Role of Chromatin Modifying Enzyme Ezh2 in CD8$^+$ T Cells

Data from previous studies demonstrate that the loss of Ezh2 in mature T cells impairs their proliferative response to anti-CD3 Ab [58]. We observed that Ezh2 mRNA and protein were significantly increased in alloreactive CD8$^+$ T_E (Figure 5B and C). Flow cytometry analysis showed at the single cell level that all day 14 CD8$^+$ T_E expressed higher levels of Ezh2 protein than T_N (Figure 5D). Further tests using MSigDBv2 demonstrated that alloreactive CD8$^+$ T_E activated 23 of 30 Ezh2 target or partner genes previously identified by others [54] (Figure 6A). Ex vivo culture confirmed that purified Ezh2-shRNA GFP$^+$CD8$^+$ T_N had reduced expression of Ezh2 protein (Figure 6B) and decreased their expansion by approximate 4-fold in response to anti-CD3 and anti-CD28 Abs compared to Control-shRNA GFP$^+$CD8$^+$ T cells (Figure 6C). Thus, Ezh2 may play important roles in antigen-activated CD8$^+$ T cells.

We further asked whether Ezh2 inhibition had differential effects on alloantigen stimulated versus homeostatic cytokine IL-7 mediated CD8$^+$ T cell proliferation. Because our previous studies suggest that purified mouse T cells rapidly diminish in cultures in the absence of DCs [30], we stimulated unfractionated Ezh2-shRNA GFP$^+$CD8$^+$ T_N with allogeneic DCs or with IL-7. Five days later, cells were recovered from the culture and analyzed for the expansion of GFP$^+$CD8$^+$ T cells using flow cytometry. Interestingly, compared

Table 3. ESC-Related Genes That Are Activated in CD8$^+$ T_E

Gene Symbol	Gene ID#	Fold Change T_{MSC}/T_N	Fold Change T_E/T_N
Cell cycle			
Ccnb1	268697	2.28	16.85
Sgol1	72415		11.04
Chek1	12649	2.6	10.34
Cks1b	54124	2.58	9.51
Cdkn1a	12575		9.17
Plk1	18817		7.54
Aspm	12316		7.53
Ect2	13605		6.86
Itgb1	16412		5.58
Ccne1	12447		5.31
Syce2	71846		5.16
Cdc25c	12532		4.72
Ccnf	12449		3.96
Dlg7	218977		2.72
Cdkn2a	12578		2.55
Mybl2	17865		2.43
Ran	19384		1.89
Ccng1	12450		1.84
Erh	13877	1.67	1.74
Psmd1	70247		1.69
Cops5	26754		1.64
DNA replication/Repair			
Rrm2	20135	9.19	60.07
Cdc6	23834	5.09	21.54
Ill1rl1	17082		19.15
Dtl	76843	4.19	18.32
Rad51	19361	2.92	15.71
Pttg1	30939		8.1
Dna2l	327762	1.68	7.48
Brca1	12189	2.04	5.87
Smc2	14211		3.55
Mcm2	17216	1.56	3.4
Rfc5	72151		2.7
Mcm3	17215		2.7
Mcm4	17217		2.59
Nudt1	17766	1.66	2.53
Orc6l	56452		2.44
Rfc4	106344		2.4
Rpa3	68240		2.22
Dtymk	21915	1.65	2.21
Hat1	107435		2.15
Brca2	12190		2.01
Blm	12144		1.97
Topbp1	235559		1.96
Ranbp5	70572		1.72
Dbf4	27214		1.69
Ube2n	93765		1.64
Nudt5	53893		1.52
Ubiquitin/Proteolysis/Proteasome			
Cdc20	107995		5.27
Capn2	12334		2.34
Xpnpep1	170750		2.32
Ide	15925		2.28
Coq3	230027	1.67	2.12
Psma1	26440		1.99
Psmb2	26445	1.55	1.78
Psmc2	19181		1.63
Psmb5	19173		1.62
Psmd6	66413		1.57
Psma3	19167		1.55
Psma5	26442		1.55
Metabolism			
Gldc	104174		23.94
Gsto1	14873	2.29	7.34
Bcat1	12035		5.54
Myo5a	17918	1.55	4.68
Asns	27053	2.95	3.87
Gcat	26912		2.71
Shmt1	20425	1.53	2.55
Uxs1	67883		2.48
Hmgcr	15357		1.96
Pfkfb1	18639		1.8
Lypla1	18777		1.79
Ywhah	22629		1.74
Acot9	56360		1.66
Soat1	20652		1.65
Nup93	71805		1.62
Kpna3	16648		1.56
Electron transport			
Nqo2	18105		3.3
Etfb	110826		3.11
Pdia6	71853	1.6	2.42
Gsr	14782		2.06
Cyp51	13121		1.94
Uqcrb	67530		1.92
Txnl1	53382		1.81
Sqle	20775		1.69
Txndc4	76299		1.6
Txnl5	52700		1.6
Chromatin modification/Assembly			
Nusap1	108907	1.86	9.22
Mad2l1	56150	1.61	6.8
Tmem38b	52076		2.7
Nek2	18005		2.45
Suv39h2	64707		2.04
Cbx1	12412		1.94
Nap1l1	53605		1.65
Smarcad1	13990		1.56
Transcription/Transcription regulation			
Mdfic	16543	2.77	18.58
Prdm1	12142		10.62
Tacc3	21335		6.06
Csda	56449		5.52
Cdkn2b	12579		4.89
Plagl1	22634		3.25
Sub1	20024		2.62
Myef2	17876		2.09
Gtf2e2	68153		1.99
Tceb1	67923		1.95
Polr3k	67005		1.77
Bzw1	66882		1.68
Cnot7	18983		1.63
Klf9	16601		1.6
Mtdh	67154		1.57
Nfyb	18045		1.52
RNA processing			
Syncrip	56403	1.79	2.88
Hnrpll	72692		2.73
Ell2	192657		2.42
Ncbp2	68092	1.9	2.01
Snrpa1	68981		1.91
Cstf3	228410		1.84
Sfrs3	20383		1.71
Cops2	12848		1.7
Lsm5	66373		1.7
Sfrs2	20382		1.65
Translation			
Farsb	23874		4.68
Mrpl27	94064	1.84	2.41
Eif4e2	26987	1.52	2.04
Eif1ay	66235		1.86
Mrpl18	67681		1.69
Eif3s1	78655		1.63
Mrpl16	94063		1.52
Protein phosphorylation			
Melk	17279	4.05	17.84
Bub1	12235		7.69
Wee1	22390	2.07	6.06
Ryk	20187		5.21
Plk4	20873	1.54	4.75
Ttk	22137		3.62
Mapk6	50772		2.07
Chek2	50883		2.07
Ppap2a	19012		2.05
Pigf	18701		1.61
Acp1	11431		1.56
Ptprk	19272		1.54
Receptor/Signal transduction			
Spp1	20750		4.52
Aurka	20878		4.47
Exo1	26909		4.02
Hif1a	15251		3.71
Csrp2	13008		3.01
Sap30	60406		2.5
Dusp16	70686		2.3
Rap2a	76108		2.27
Bax	12028		1.84
Pex7	18634	1.6	1.74
Bdnf	12064		1.72
Gnb1	14688		1.69
Hnrpab	15384		1.65
Ddx1	104721		1.65
Inhbb	16324		1.64
Nasp	50927		1.61
Sfrs1	110809		1.61
Snrpd1	20641		1.55
Hrb	15463		1.55
Rab1	19324		1.53
Vdac3	22335		1.52
Transport (Protein/Ion)			
Kif20a	19348		4.26
Arl5a	75423		3.8
Snx10	71982	1.97	2.74
Clic4	29876	2.31	2.56
Slc35b1	110172		2.15
Arl1	104303		2.07
Lman1	70361		1.98
Vps54	245944		1.94
Mtch2	56428		1.86
Snx5	69178	1.77	1.85
Clcn5	12728		1.85
Slc2a3	20527		1.83
Nup62	18226		1.78
Slc33a1	11416		1.72
Golt1b	66964		1.7
Slc4a7	218756		1.69
Bcap29	12033	1.61	1.65
Slc12a2	20496		1.57
Inhibitorysignal/Apoptosis			
Ckap2	80986		5.44
Gas2	14453		4.73
Cell adhesion/Communication			
Adam19	11492		4.89
Adam9	11502		2.82
Tnc	21923	1.94	1.56
Vcl	22330		1.66
Microtubule-based process			
Prc1	233406	1.86	12.05
Cks2	66197	4.85	11.65
Anln	68743		4.82
Dstn	56431		1.74
Cytokinesis			
Igfbp7	29817		2.68
Wisp1	22402		2.26
Miscellaneous			
Fign11	60530	3.2	15.73
Trip13	69716	1.88	8.69
Pcbp4	59092		6.39
Tmem49	75909	2.26	6.27
Tacstd1	17075		5.17
Smoc2	64074		4.05
Synpo	104027	1.75	3.68
Tipin	66131		3.5
Ccdc99	70385		2.81
Lactb2	212442	1.64	2.68
Gmfb	63985	1.67	2.64
Wbp5	22381		2.42
Errfi1	74155	1.54	2.4
Ccdc80	67896		2.34
Nucks1	98415		2.3
Mpdu1	24070	1.85	2.29
Ipp	16351		2.25
Pls3	102866		2.16
Phlda3	27280		2.1
Dsp	109620		1.96
Prdx1	18477		1.91
Tmem41b	233724		1.83
Rcn1	19672	1.6	1.77
Esd	13885		1.73
Psmc3ip	19183		1.71
Ranbp1	19385		1.67
Nup85	445007		1.62
Ppp1r7	66385		1.62
Psmd7	17463	1.84	1.54
Cox4nb	18117		1.54
Asah1	11886		1.53
Uck2	80914	1.74	1.52
Tiprl	226591		1.51

Table 4. NSC-Related Genes That Are Activated in CD8$^+$ T_E

		Fold Change				Fold Change				Fold Change				Fold Change		
Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N
Cell cycle				**DNA replication/Repair**				**Receptor/Signal transduction**				**Transport (Protein/Ion)**				**Transcription/Transcription regulation**			
Ccnb2	12442	3.64	29.94	Rrm2	20135	9.19	60.07	Epas1	13819		7.54	Sypl	19027		4.33	Mdfic	16543	2.77	18.58
Ccna2	12428	3.31	21.13	Cdc6	23834	5.09	21.54	Rhoc	11853		6.85	Kif20a	19348		4.26	Uhrf1	18140	2.89	9.26
Cdca5	67849	4.46	19.44	Dtl	76843	4.19	18.32	Rhoq	104215	1.69	5.32	Syt11	229521	2.9	3.88	Hells	15201	1.91	7
Ccnb1	268697	2.28	16.85	Uhrf1	18140	2.89	9.26	Arl5a	75423		3.8	Snx10	71982	1.97	2.74	Ezh2	14056		6.95
Sgol1	72415		11.04	Dna2l	327762	1.68	7.48	Hif1a	15251		3.71	Slc35b2	73836		2.61	Tacc3	21335		6.06
E2f8	108961		10.39	Lig1	16881		5.73	Nudt1	17766	1.66	2.53	Clic4	29876	2.31	2.56	Eomes	13813	1.61	5.7
Chek1	12649	2.6	10.34	Rpa2	19891		4.52	Sap30	60406		2.5	Pdzd11	72621	1.66	2.25	Csda	56449		5.52
Cks1b	54124	2.58	9.51	Rfc3	69263	1.6	4.27	Dusp16	70686		2.3	Slc35b1	110172		2.15	Cenpk	60411		4.51
Cdkn1a	12575		9.17	Rrm1	20133		4.18	Dtymk	21915	1.65	2.21	Dbi	13167		2.11	Nsbp1	50887		3.85
Plk1	18817		7.54	Mcm5	17218		4.07	Gpr56	14766		2.08	Lman1	70361		1.98	Klf10	21847		2.98
Aspm	12316		7.53	Smc2	14211		3.55	Akt1	11651		2.07	Vps54	245944		1.94	Wdhd1	218973		2.85
Ect2	13605		6.86	Fen1	14156		3.43	Arl1	104303		2.07	Gipc1	67903	1.55	1.93	Sub1	20024		2.62
Cdkn2c	12580	2.13	6.27	Prim1	19075		3.42	Arhgap21	71435		2	Arf4	11843		1.87	Pmf1	67037		2.45
Itgb1	16412		5.58	Mcm2	17216	1.56	3.4	Tank	21353		1.95	Mtch2	56428		1.86	Myef2	17876		2.09
Gmnn	57441	2.1	5.33	Hmgn2	15331		3.37	Fut8	53618		1.91	Snx5	69178	1.77	1.85	Rab8b	235442		2.09
Cdc45l	12544	1.68	5.13	Mcm7	17220		2.94	Rbl1	19650		1.83	Clcn5	12728		1.85	Gtf2e2	68153		1.99
Cdkn2b	12579		4.89	Prim2	19076		2.76	Tmpo	21917		1.82	Vps29	56433		1.83	Inpp1l	16332		1.98
Cdc25c	12532		4.72	Rfc5	72151		2.7	Ptprj	19271		1.74	Rrbp1	81910		1.8	Cdca7l	217946	1.8	1.97
Pola1	18968	1.59	4.55	Mcm3	17215		2.7	Csnk2b	13001		1.73	Trappc1	245828		1.79	Tceb1	67923		1.95
Aurka	20878		4.47	Mcm4	17217		2.59	Ranbp5	70572		1.72	Ssr2	66256		1.73	Hnrpdl	50926	1.72	1.78
Ccnf	12449		3.96	Orc6l	56452		2.44	Gnb1	14688		1.69	Slc33a1	11416		1.72	Polr3k	67005		1.77
Gsg2	14841	1.75	3.11	Rfc4	106344		2.4	Dck	13178		1.66	Vps45	22365		1.72	Bzw1	66882		1.68
Dlg7	218977		2.72	Recql	19691	1.71	2.28	Ddx1	104721		1.65	Atp6v0a2	21871		1.71	Cnot7	18983		1.63
Nfatc1	18018		2.51	Rpa3	68240		2.22	Inhbb	16324		1.64	Slc4a7	218756		1.69	Sin3b	20467		1.63
Ran	19384		1.89	Smc6	67241		2.15	Tyms	22171		1.63	Slc39a6	106957		1.65	Mtdh	67154		1.57
Ccng1	12450		1.84	Hat1	107435		2.15	Aaas	223921		1.59	Bcap29	12033	1.61	1.65	Th1l	57314		1.53
Erh	13877	1.67	1.74	Blm	12144		1.97	Smap1	98366		1.58	Nup93	71805		1.62	**Microtubule-based process**			
Rb1	19645		1.72	Topbp1	235559		1.96	Rab1	19324		1.53	Sfxn1	14057	1.7	1.57	Kif2c	73804	3.23	20.24
Psmd1	70247		1.69	Hmgb1	15289		1.82	Asna1	56495		1.53	Timm17b	21855		1.57	Kif11	16551	1.82	7.9
Cops5	26754		1.64	Ube2n	93765		1.64	Nudt5	53893		1.52	Kpna3	16648		1.56	Kif22	110033	2.33	7.45
Mapre1	13589		1.6	Smarcad1	13990		1.57	**Cytokinesis**				Slc25a10	27376		1.56	Kif4	16571		3.58
Rap1a	109905		1.53					Prc1	233406	1.86	12.05	Kdelr2	66913		1.56	Mid1ip1	68041		2.07
								Cks2	66197	4.85	11.65	Sec14l1	74136		1.54	Nde1	67203		2.04
								Incenp	16319		4.68					Kif23	71819		1.68
Metabolism				**Ubiquitin/Proteolysis/Proteasome**				Skap2	54353	1.76	2.09	**Chromatin modification/Assembly**				Pnkd	56695		2.29
Gldc	104174		23.94	Cdc20	107995		5.27	**Protein phosphorylation**				Nusap1	108907	1.86		Mpdu1	24070	1.85	2.29
Brca1	12189	2.04	5.87	Capn2	12334		2.34	Melk	17279	4.05	17.84	Mad2l1	56150	1.61		Ipp	16351		2.25
Gpd2	14571		4.17	Xpnpep1	170750		2.32	Bub1	12235		7.69	Asf1b	66929			Tmem97	69071		2.24
Lycat	225010		3.06	Ide	15925		2.28	Wee1	22390	2.07	6.06	Nek2	18005			Pls3	102866		2.16
Gcat	26912		2.71	Psma1	26440		1.99	Ddr1	12305	2.26	5.82	Cbx5	12419			Dek	110052		2.16
Azin1	54375		2.29	Psmb1	19170		1.88	Stk39	53416	1.51	5.32	Pvt1	19296	1.57		Lancl2	71835		2.15
Pkm2	18746		2.19	Ube2l3	22195		1.82	Ryk	20187		5.21	Suv39h2	64707			Pqlc3	217430		2.14
Idh3a	67834		2	Blmh	104184		1.71	Plk4	20873	1.54	4.75	Cbx1	12412			Phlda3	27280		2.1
Gyg	27357		1.98	Ube2t	67196		1.64	Ttk	22137		3.62	Nap1l1	53605			Pih1d1	68845	1.59	2.03
Hmgcr	15357		1.96	Psmb4	19172		1.64	Mapk6	50772		2.07	Translation				Anp32e	66471		1.96
Rpe	66646		1.86	Psmc2	19181		1.63	Cdk2	12566		1.87	Farsb	23874		4.68	Tmem30a	69981		1.92

(*Continued*)

Table 4. (*Continued*)

		Fold Change				Fold Change				Fold Change					Fold Change				Fold Change	
Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N	Gene Symbol	Gene ID#	T_{MSC}/T_N	T_E/T_N
Sdhb	67680		1.8	Ube2e3	22193		1.63	Commd1	17846		1.81	Lamp2	16784	2.63	3.48	Prdx1	18477		1.91
Acsl5	433256		1.79	Psmb5	19173		1.62	Ptp4a2	19244		1.74	Mrpl27	94064	1.84	2.41	Mlf2	30853		1.88
Lypla1	18777		1.79	Psmd6	66413		1.57	Rps6ka1	20111		1.73	Eif4e2	26987	1.52	2.04	Hccs	15159		1.85
Decr1	67460		1.79	Psma3	19167		1.55	Ptpn9	56294		1.59	Eif1ay	66235		1.86	Actb	11461		1.84
Gstt1	14871		1.78	Psma5	26442		1.55	Ppp5c	19060	1.53	1.58	Mrpl13	68537		1.71	Tex9	21778		1.8
Ywhah	22629		1.74	**RNA processing**				Acp1	11431		1.56	Mrpl18	67681		1.69	Rcn1	19672	1.6	1.77
Gusb	110006		1.72	Syncrip	56403	1.79	2.88	**Inhibitory signal/Apoptosis**				Eif3s1	78655		1.63	Nubp1	26425	1.59	1.74
Soat1	20652		1.65	Slbp	20492		2.76	Lgals1	16852	3.91	27.4	Mrpl16	94063		1.52	Psmc3ip	19183		1.71
Aytl2	210992		1.65	Hnrpll	72692		2.73	Birc5	11799	5.33	21.6	**Miscellaneous**				Pdap1	231887		1.69
Aco1	11428		1.58	Thoc4	21681		2.21	Casp3	12367		5.37	Fign11	60530	3.2	15.73	Arpc5	67771		1.67
Pcyt1a	13026		1.57	Cpsf2	51786		2.02	Gas2	14453		4.73	Trip13	69716	1.88	8.69	Ranbp1	19385		1.67
											2.37	Pcbp4	59092						
Electron transport				Ncbp2	68092	1.9	2.01	Dap	223453						6.39	Tmem77	67171		1.65
Acadl	11363		5.27	Snrpa1	68981		1.91	Bcl7c	12055		2.23	Sh3bgrl	56726		4.77	Pon2	330260		1.65
Pdia6	71853	1.6	2.42	Cstf3	228410		1.84	Api5	11800		2.16	Lxn	17035		4.32	Nup85	445007		1.62
Gsr	14782		2.06	Rod1	230257		1.79	Casp7	12369		2.14	Capg	12332	1.95	4.17	Ppp1r7	66385		1.62
Cyp51	13121		1.94	Sfrs3	20383		1.71	Phlda1	21664		1.94	Ltb4dh	67103		4.01	Sephs2	20768		1.61
Uqcrb	67530		1.92	Cops2	12848		1.7	Bax	12028		1.84	Gtse1	29870		3.59	Zdhhc6	66980		1.59
Glrx	93692		1.87	Lsm5	66373		1.7	Bnip2	12175		1.51	Tipin	66131		3.5	Pdia3	14827		1.58
Txnl1	53382		1.81	Sfrs2	20382		1.65	**Cell adhesion/Communication**				Ccdc99	70385		2.81	Mgat2	217664		1.56
Cyb5b	66427		1.7	Sfrs1	110809		1.61	Itgb1	16412		5.58	Mtm1	17772		2.71	Psmd7	17463	1.84	1.54
Sqle	20775		1.69	**Regulation of cell growth/Proliferation**				Adam19	11492		4.89	Wbp5	22381		2.42	Psmc1	19179		1.54
Txndc4	76299		1.6	Scin	20259		3.06	Adam9	11502		2.82	Acyp2	75572	1.75	2.41	Asah1	11886		1.53
Cyb5r3	109754		1.57	Chpt1	212862		2.36	Tnc	21923	1.94	1.56	Nucks1	98415		2.3	Peci	23986		1.53

Table 5. Functional Categories of 401 CD8$^+$ T_E-Related Stem Cell Genes (See Gene Symbols and Identification# in Table 3 and Table 4)

Category	Gene Symbols
1. Cell cycle regulation	
Cell cycle (35)	Ccna2, Cdca5, Ccnb1, Sgol1, E2f8, Chek1, Cks1b, Cdkn1a, Ncaph, Plk1, Aspm, Ect2, Cdkn2c, Gmnn, Ccne1, Syce2, Cdc45l, Cdkn2b, Cdc25c, Pola1, Aurka, Ccnf, Gsg2, Dlg7, Cdkn2a, Nfatc1, Mybl2, Ran, Ccng1, Erh, Rb1, Psmd1, Cops5, Mapre1, Rap1a
2. Resistance to stress	
DNA replication/repair (34)	Rrm2, Cdc6, Il1rl1, Dtl, Rad51, Uhrf1, Pttg1, Dna2l, Lig1, Rpa2, Rfc3, Rrm1, Mcm5, Smc2, Fen1, Prim1, Mcm2, Hmgn2, Mcm7, Prim2, Mcm3, Rfc5, Mcm4, Orc6l, Rfc4, Recql, Rpa3, Smc6, Brca2, Blm, Topbp1, Hmgb1, Dbf4, Ube2n
Ubiquitin/proteolysis/proteasome (20)	Cdc20, Coq7, Nedd4, Capn2, Xpnpep1, Ide, Coq3, Psma1, Psmb1, Ube2l3, Psmb2, Blmh, Ube2t, Psmb4, Psmc2, Psmb5, Psmd6, Psma3, Psma5, Ube2e3
Metabolism (27)	Gldc, Gsto1, Brca1, Bcat1, Myo5a, Gpd2, Lycat, Gcat, Shmt1, Uxs1, Azin1, Pkm2, Idh3a, Gyg, Hmgcr, Rpe, Pfkfb1, Sdhb, Decr1, Acsl5, Lypla1, Gstt1, Gusb, Acot9, Aytl2, Soat1, Aco1
Electron transport (14)	Acadl, Nqo2, Etfb, Pdia6, Gsr, Cyp51, Uqcrb, Glrx, Txnl1, Cyb5b, Sqle, Txndc4, Txnl5, Cyb5r3
3. Chromatin modification, and transcription/translation regulation	
Chromatin modification/assembly (12)	Nusap1, Mad2l1, Asf1b, Tmem38b, Nek2, Cbx5, Pvt1, Hat1, Suv39h2, Cbx1, Nap1l1, Smarcad1
Transcription/transcription regulation (33)	Mdfic, Prdm1, Uhrf1, Hells, Ezh2, Tacc3, Eomes, Csda, Cenpk, Nsbp1, Klf10, Wdhd1, Sub1, Sap30, Pmf1, Myef2, Rab8b, Gtf2e2, Inppl1, Cdca7l, Tceb1, Rbl1, Tmpo, Hnrpdl, Polr3k, Cops2, Bzw1, Sin3b, Cnot7, Klf9, Mtdh, Th1l, Nfyb
RNA processing (16)	Syncrip, Slbp, Hnrpll, Ell2, Thoc4, Cpsf2, Ncbp2, Snrpa1, Cstf3, Rod1, Sfrs3, Lsm5, Sfrs2, Ddx1, Sfrs1, Snrpd1
Translation (11)	Farsb, Lamp2, Mrpl27, Eif4e2, Lamc1, Ap3s1, Mrpl13, Mrpl18, Eif3s1, Dnajc9, Mrpl16
4. Survival/apoptosis	
Survival/apoptosis (16)	Lgals1, Birc5, Ckap2, Casp3, Gas2, Scin, Grn, Dap, Bcl7c, Api5, Casp7, Skap2, Phlda1, Bax, Nasp, Bnip2
5. Signaling	
Protein phosphorylation (20)	Melk, Bub1, Wee1, Ddr1, Stk39, Ryk, Plk4, Ttk, Mapk6, Chek2, Ppap2a, Cdk2, Commd1, Ptp4a2, Rps6ka1, Pigf, Ptpn9, Ppp5c, Acp1, Ptprk
Receptor/signal transduction (24)	Epas1, Rhoc, Rhoq, Hif1a, Grn, Dusp16, Rap2a, Gpr56, Akt1, Arhgap21, Tank, Gipc1, Fut8, Rrbp1, Csnk2b, Ssr2, Ranbp5, Gnb1, Nup93, Nasp, Smap1, Timm17b, Kpna3, Rab1
6. Others	
Transport (protein/ion) (33)	Sypl, Kif20a, Syt11, Arl5a, Snx10, Slc35b2, Clic4, Slc35b1, Dbi, Arl1, Lman1, Vps54, Arf4, Mtch2, Clcn5, Snx5, Vps29, Slc2a3, Trappc1, Nup62, Slc33a1, Vps45, Atp6v0a2, Golt1b, Slc4a7, Bcap29, Slc39a6, Aaas, Sfxn1, Slc12a2, Slc25a10, Sec14l1, Asna1
Cell adhesion/communication (5)	Itgb1, Adam19, Adam9, Vcl, Tnc
Microtubule-based process/ cytokinesis (12)	Kif2c, Kif11, Kif22, Kif4, Mid1ip1, Nde1, Kif23, Prc1, Cks2, Anln, Incenp, Dstn
Miscellaneous (89)	Wisp1, Igfbp7, Chpt1, Fign11, Trip13, Pcbp4, Tmem49, Tacstd1, Sh3bgrl, Spp1, Lxn, Capg, Smoc2, Exo1, Ltb4dh, Asns, Synpo, Gtse1, Tipin, Plagl1, Ccdc99, Mtm1, Lactb2, Gmfb, Nudt1, Wbp5, Acyp2, Errfi1, Htatip2, Ccdc80, Nucks1, Pnkd, Mpdu1, Ipp, Tmem97, Dtymk, Apaf1, Pls3, Dek, Lancl2, Pqlc3, Fyn, Phlda3, Pih1d1, Anp32e, Dsp, Tmem30a, Prdx1, Mlf2, Hccs, Actb, Tmem41b, Tex9, Rcn1, Ptprj, Nubp1, Pex7, Ywhah, Esd, Bdnf, Psmc3ip, Nab1, Pdap1, Arpc5, Ranbp1, Dck, Hnrpab, Tmem77, Pon2, Inhbb, Tyms, Nup85, Ppp1r7, Sephs2, Zdhhc6, Pdia3, Pcyt1a, Mgat2, Kdelr2, Hrb, Psmd7, Psmc1, Cox4nb, Asah1, Peci, Uck2, Nudt5, Vdac3, Tiprl

T_E indicates effector T cells;

to Control-shRNA GFP$^+$CD8$^+$ T cells, the expansion of Ezh2-shRNA GFP$^+$CD8$^+$ T cells was decreased in the culture supplemented with allogeneic DCs but not with IL-7 (Figure 6D). This suggests that Ezh2 may be required for antigen-driven T cell responses rather than homeostatic T cell proliferation.

DISCUSSION

These studies identify a group of stem cell genes that are normally expressed in ESCs and NSCs in alloreactive CD8$^+$ T cells. Most of these stem cell genes are found to be essential to cell cycle regulation, DNA replication and repair, stress resistance, chromatin modification, and transcription regulation. One of these genes, Ezh2, emerges as an important regulator for the proliferation of antigen-activated CD8$^+$ T cells. On the other hand, alloreactive CD8$^+$ T_E increase the expression of many genes that mediate cell apoptosis and growth arrest. We demonstrate that these alloreactive CD8$^+$ T_E were rapidly diminished in vivo in lethally irradiated secondary congenic recipients, suggesting that homeostatic factors alone are not sufficient to sustaining alloreactive CD8$^+$ T_E. However, upon chronic exposure to alloantigens alloreactive CD8$^+$ T_E proliferate to persist in vivo despite their massive apoptotic death. These data indicated that although alloreactive CD8$^+$ T_E were "terminally differentiated" with drastically increased susceptibility to apoptotic death, they had the ability to survive and persist via the mechanisms of continuous replication in the presence of alloantigens. Thus, these newly identified stem cell genes could be important targets for understanding and modulating allogeneic T cell responses and GVHD.

T cells are known to be stem cell-like cells [1,3,4]. Gene expression profile analysis reveals that memory T cells may trigger HSC-related transcriptional programs to regulate their self-renewal in the absence of antigens [17]. We found that alloreactive CD8$^+$ T_E

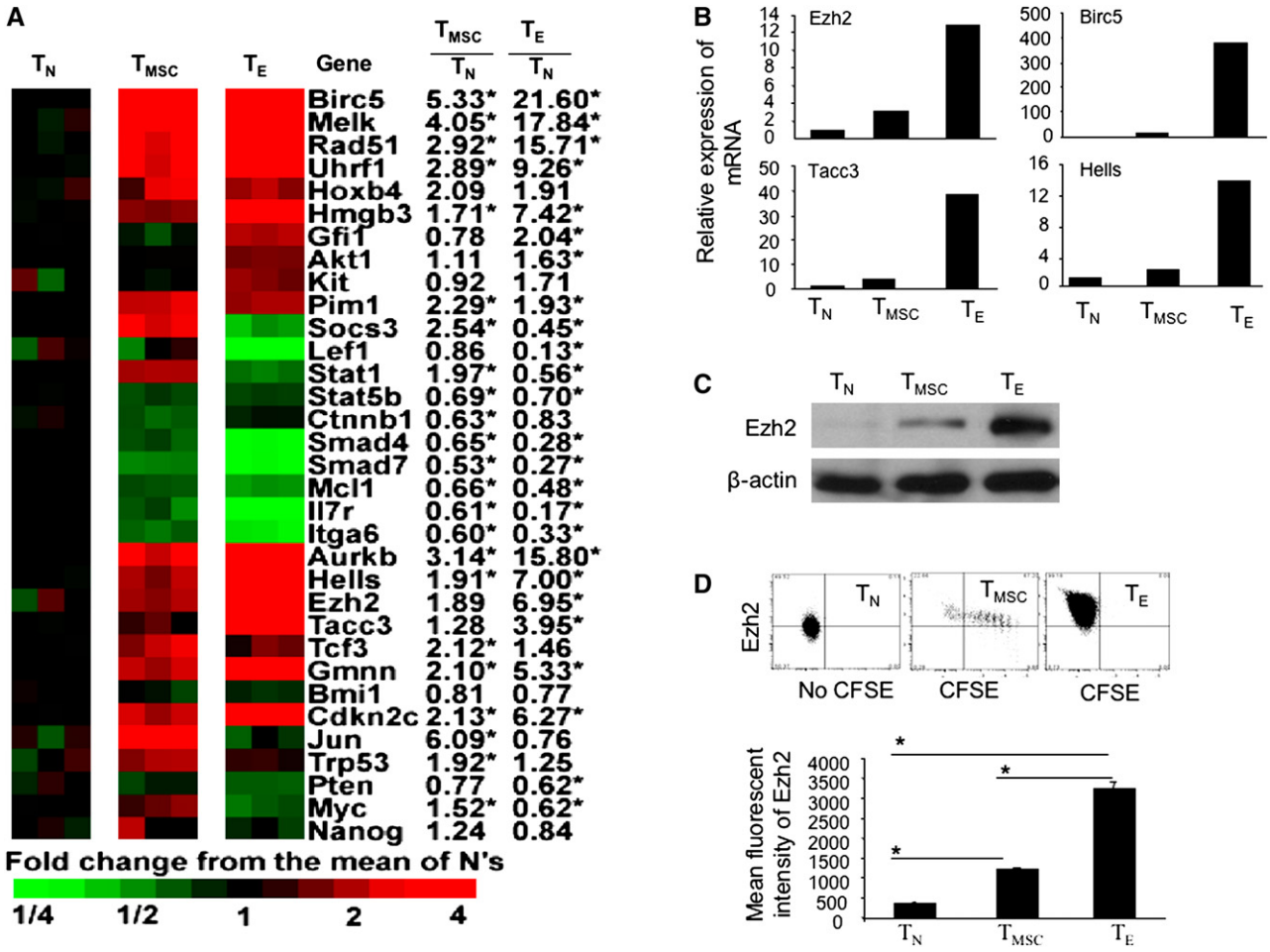


Figure 5. Characterization of stem cell genes in alloreactive CD8$^+$ T cells. (A) The relative expression of stem cell genes in alloreactive CD8$^+$ T_E and CD8$^+$ T_{MSC} are shown with genes identified by functional set enrichment analysis using curated gene lists from MSigDBv2 (Figure 4). (B) Real-time RT-PCR analysis shows the relative mRNA expression of selected genes in each T cell subset. Data are representative of 3 independent preparations of alloreactive T cells. (C) Western blot analysis shows the expression of EZH2 protein in purified day 14 CD8$^+$ T_E, T_{MSC}, and control T_N. (D) donor CD8$^+$ T_N and day 14 CD8$^+$ T cells were stained with anti-Ezh2 Ab using intracellular staining and analyzed by flow cytometry. Dot plots show the expression of Ezh2 in cell subset of donor T cells. Mean fluorescein intensity shows the amount of testing antigen.

activated stem cell transcriptional programs are operational in both ESCs and NSCs, whereas genes decreased in CD8$^+$ T_E were overrepresented in HSC-enriched genes. Our observations were further validated by our reanalyzing the gene expression profile of Sarkar et al. [42], which revealed that ESC- and NSC-related genes were activated in LCMV gp33-specific CD8$^+$ T_E, but decreased in CD8$^+$ memory T cells. It appears that functional T cell subsets can be defined by different stem cell transcriptional programs that regulate their unique properties of T_E versus memory T cells. It is likely that alloreactive CD8$^+$ T_E require ESC- and NSC-related transcriptional programs to generate a large number of functionally active effectors sufficient to eliminate the target antigen.

Interestingly, reactivation of stem cell transcriptional programs occurs in both alloantigen-specific CD8$^+$ T_E generated during GVHD and viral antigen-reactive CD8$^+$ T_E derived from acute infection. We identified these CD8$^+$ T_E-related stem cell transcriptional programs using curated gene lists from MSigDBv2 [40]. Although it has been reported that stem cell transcriptional profiles identified by different groups do not correlate well with each other [59], we found that approximately 30% of the genes increased in our alloreactive CD8$^+$ T_E are present on ESC and/or NSC gene lists of Ramalho-Santos et al. [41]. Other studies also clearly showed a significant correlation of stem cell gene expression profiles identified by different groups [41,60]. Furthermore, we independently validated that the gene expression profiles of our alloreactive CD8$^+$ T_E were correlated significantly with those P14 CD8$^+$ T_E of Sarkar et al. [42]. Thus, these CD8$^+$ T_E-related stem cell transcriptional programs may have significant implications in regulating T cells across various types of immune responses.

Overlapped gene expression profiles between CD8$^+$ T_E and embryonic and neural stem cells suggest

Table 6. HSC-Enriched Genes That Are Decreased in CD8$^+$ T_E

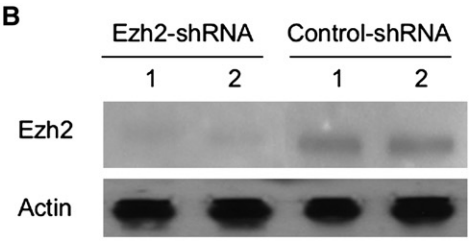
Gene Symbol	Gene ID#	Fold Change T_{MSC}/T_N	Fold Change T_E/T_N
Cell cycle			
Lats2	50523		−1.6
Rassf2	215653		−1.64
Flcn	216805		−2.1
Sesn1	140742	−2.71	−5.69
DNA replication/Repair			
Polg	18975		−1.55
E4f1	13560		−1.63
Sfpq	71514	−1.54	−1.75
Xpc	22591		−2.52
Nfix	18032		−6.12
Ubiquitin/Proteolysis/Proteasome			
Usp3	235441		−1.54
Fbxo38	107035		−1.57
Ubl4	27643		−1.62
Usp9x	22284		−1.64
Fbxo9	71538		−1.72
Usp19	71472	−1.66	−1.85
Rnpepl1	108657	−1.56	−2.04
Ube2d2	56550	−1.77	−2.06
Rbbp6	19647	−1.5	−2.1
Zfp292	30046		−2.31
Rffl	67338		−2.35
Klk8	259277		−4.21
Metabolism			
Tk2	57813		−1.5
Glb1l	74577		−1.51
Idua	15932		−1.52
Glul	14645		−1.54
Elovl6	170439		−1.55
Supt6h	20926		−1.61
Pcyt2	68671		−1.69
Mthfr	17769		−1.71
Ndufa6	67130		−1.85
Pgs1	74451		−2.1
Ihpk1	27399		−2.26
Cbr1	12408		−2.55
Lpin1	14245		−2.57
Man2c1	73744		−2.88
Gstk1	76263		−2.99
Adcy6	11512		−3.08
Dph5	69740		−3.81
Ldhb	16832	−1.97	−4.83
Pdk1	228026		−6.29
RNA processing			
Rpusd4	71989		−1.51
Imp3	102462		−1.54
Mphosph10	67973		−1.62
Prpf38b	66921		−1.65
Nola3	66181		−1.8
Utp20	70683		−2.32
Translation			
Mknk2	17347		−1.62
Mrpl24	67707		−1.75
Eif4g2	13690	−1.55	−1.8
Rpl37a	19981		−2.18
Rpl13	270106		−2.57
Rpl22	19934		−2.59
Protein phosphorylation			
Tesk1	21754		−1.59
Mast3	546071		−1.59
Prkcq	18761		−1.64
Jak1	16451	−1.52	−1.75
Csnk1g2	103236		−1.75
Ptpn21	24000	−1.68	−1.87
Prkce	18754		−1.96
Map4k4	26921		−2.44
Tec	21682		−2.47
Ikbke	56489		−2.52
Ptpn23	104831		−2.87
Prkd2	101540		−2.98
Transcription/Transcription regulation			
Zzz3	108946		−1.51
Zmynd11	66505		−1.51
Zkscan6	52712		−1.53
Nfe2l1	18023		−1.53
Ezh1	14055		−1.55
Scmh1	29871		−1.56
Zkscan3	72739		−1.6
Zkscan1	74570		−1.69
Rarg	19411	−2.56	−1.73
Zfx	22764		−1.73
Mef2d	17261		−1.77
Ncor2	20602	−1.6	−1.81
Zfml	18139	−1.79	−2.05
Zfp96	22758		−2.06
Zeb1	21417		−2.11
Taf1a	21339		−2.28
Bach1	12013	−1.55	−2.34
Klf3	16599		−2.52
Jmjd1a	104263		−2.54
Basp1	70350	−2.71	−2.64
Ash1l	192195	−1.83	−2.84
Zfp1	22640		−2.91
Zbtb20	56490	−2.61	−3.08
Dbp	13170	−1.96	−3.63
Smad7	17131	−1.87	−3.68
Irf6	54139	−2.29	−4.63
Ssbp2	66970		−6.88
Chromatin modification/Assembly			
Chd1	12648		−1.59
Smarca2	67155		−2.44
Receptor/Signal transduction			
Aplp2	11804	−1.75	−1.5
Prpf6	68879		−1.5
Mettl3	56335		−1.51
Notch1	18128		−1.51
Ikbkg	16151		−1.52
Chd8	67772		−1.52
Rasa3	19414		−1.54
Mapk14	26416	−1.55	−1.54
Mfng	17305		−1.56
Frap1	56717		−1.57
Itpkb	320404	−1.81	−1.59
Xab2	67439		−1.59
Dtx2	74198	−1.77	−1.6
Dicer1	192119	−1.77	−1.63
Srpk2	20817	−1.53	−1.68
Mapk8	26419	−1.58	−1.73
Stat6	20852		−1.74
Ppp2r5e	26932		−1.76
Phc2	54383		−1.78
Rrad	56437		−1.84
Stub1	56424		−1.84
Arrb1	109689		−1.87
Azi2	27215	−1.74	−1.91
Rai1	19377	−1.61	−1.91
Mfhas1	52065		−1.98
Foxo3a	56484		−1.99
Spred2	114716		−2.07
Gaa	14387		−2.13
Dvl1	13542		−2.15
Il16	16170	−1.64	−2.26
Spsb1	74646		−2.26
Gbp2	14469		−2.29
Crebbp	12914	−1.64	−2.41
Macf1	11426	−1.61	−2.57
Il4ra	16190		−2.8
Ltb	16994		−2.81
Eng	13805		−3.43
Smad4	17128	−1.53	−3.53
Il6st	16195	−1.61	−4.31
Smad1	17125		−5.01
Mettl4	76781	−1.58	−5.5
Notch2	18129	−1.58	−5.62
Thra	21833		−5.8
Inadl	12695		−6.08
Il6ra	16194		−18.59
Cell adhesion/Communication			
Nisch	64652	−1.74	−2.16
Itga6	16403	−1.67	−3.24
Regulation of cell growth/Proliferation			
Ppan	235036		−1.6
Creg1	433375		−2.03
Pctk1	18555		−2.09
Epc1	13831	−2.03	−2.28
Socs3	12702		−2.41
Negative regulation of transcription from RNA polymerase II promoter			
Cutl1	13047		−1.52
Id3	15903		−1.77
Bcl6	12053		−1.85
Tcf25	66855		−2.02
Tle1	21885		−3.63
Foxp1	108655	−1.67	−4.93
Transport (Protein/Ion)			
Mcfd2	193813		−1.52
Bet1l	54399		−1.59
Abcb8	74610		−1.61
Acox1	11430		−1.65
Ecgf1	72962		−1.65
Pitpnm1	18739		−1.76
Acadm	11364		−1.82
Mybbp1a	18432		−1.89
Pitpnc1	71795	−1.53	−1.97
Scamp1	107767	−1.59	−1.99
Hcn3	15168		−2.12
P2rx4	18438		−2.44
Ramp3	56089		−2.54
Laptm4b	114128	−1.88	−2.88
Kcnn4	16534		−3.1
Rab3ip	216363		−3.12
Slco3a1	108116		−3.18
Slc12a7	20499		−4.35
Abca1	11303		−4.42
Ramp1	51801	−1.68	−17.13
Miscellaneous			
Tnip1	57783	−1.69	−1.51
Nphp1	53885		−1.52
Isca1	69046		−1.53
Msl2l1	77853		−1.54
Nol1	110109		−1.56
Dctn1	13191		−1.56
Klhl7	52323		−1.62
Sh3gl1	20405		−1.63
Mgea6	217615		−1.65
Bcl7b	12054		−1.66
Gltscr2	68077		−1.7
Serf2	378702		−1.7
Dhx30	72831		−1.71
Cep350	74081	−1.57	−1.74
Cramp1l	57354	−1.78	−1.74
Camsap1	227634		−1.75
Las1l	76130		−1.76
Pcf11	74737		−1.78
Serpini1	20713		−1.79
Fnbp4	55935		−1.81
Ccdc53	67282		−1.85
Phf1	21652		−1.86
Gtf2ird2	114674		−1.87
Numa1	101706	−1.57	−1.88
Brd1	223770		−1.9
Plekha1	101476		−1.91
Frmd6	319710	−1.93	−1.98
Akap8l	54194		−1.98
Grwd1	101612		−1.99
Otud5	54644		−1.99
Etnk1	75320		−2.02
Abcf3	27406		−2.03
Acyp1	66204		−2.04
Nsun4	72181		−2.06
Tmem141	51875		−2.07
Snord22	83673		−2.07
Lrrc8a	241296	−1.6	−2.1
Paqr7	71904	−1.68	−2.1
Cdc42se2	72729	−1.56	−2.12
Bola2	66162		−2.16
Crlf3	54394	−1.55	−2.21
Zdhhc9	208884		−2.26
Gas5	14455		−2.3
Tmem66	67887		−2.33
Rbm38	56190		−2.39
Jarid1b	75605		−2.4
Marveld1	277010		−2.47
Ctdsp2	52468	−1.94	−2.56
Armcx2	67416		-2.68
Ipo4	75751		−2.82
Rabac1	14470		−3.3
Plekho1	67220		−3.54
Peli1	67245	−1.94	−4.03
Btbd14a	67991		−4.63
Rreb1	68750		−7.28
Cxxc5	67393	−2.79	−7.61

HSC indicates hematopoietic stem cells.

A

Ezh2 target genes in CD8⁺ T_E

Gene Name	Fold changes T_E/T_N	Gene Name	Fold changes T_E/T_N
Ccnb2	29.9	Prim1	3.4
Top2a	20.5	Mcm2	3.4
Cdca5	19.4	Mcm3	2.7
Aurkb	15.8	Mcm4	2.6
Prc1	12.0	Nek2	2.5
Nusap1	9.2	Pcna	2.5
Ect2	6.9	Rpa3	2.2
Mad2l1	6.8	Hat1	2.2
Tacc3	6.1	Topbp1	2.0
Gmnn	5.3	Rbl1	1.8
Rrm1	4.2	Nap1l1	1.7
Cdca7	4.0		



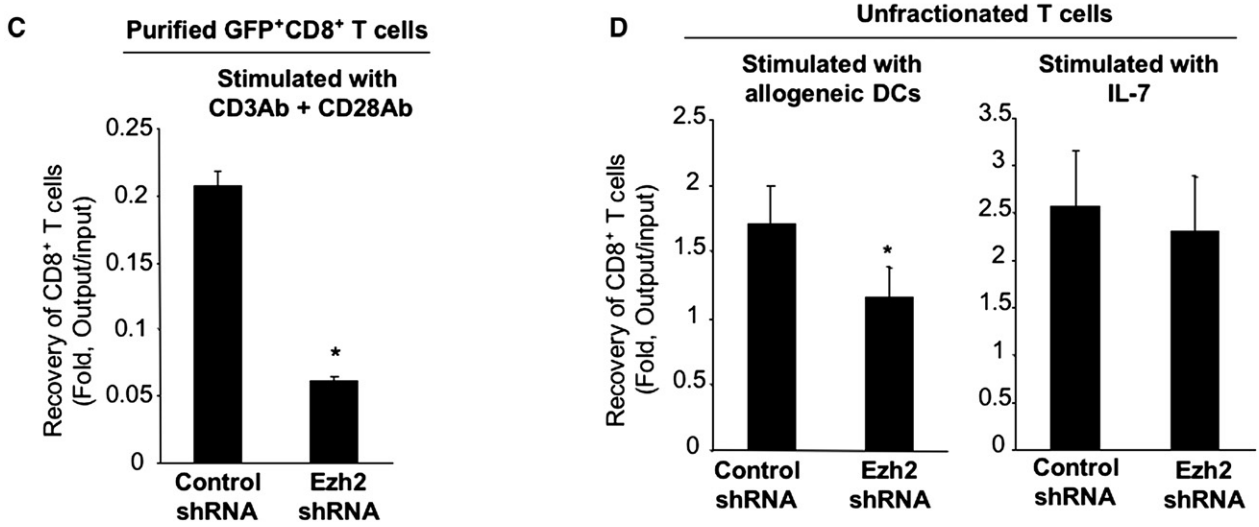


Figure 6. Identification of stem cell gene Ezh2 in alloreactive $CD8^+$ T cells. (A) The relative expression of 23 Ezh2 target or partner genes that were also selected as increased in alloreactive $CD8^+$ T_E relative to T_N. (B) Western blot shows Ezh2 protein in $CD8^+$ T_N expressing with Ezh2-shRNA or Control-shRNA. These Ezh2-shRNA GFP^+CD8^+ T_N were derived from B6 mice reconstituted with HSCs infected by Ezh2-shRNA/GFP-pLVPT^{off}. GFP^+CD8^+ T_N from B6 mice reconstituted with Con-shRNA/GFP-pLVPT^{off} were isolated as controls. (C) Sorted Ezh2-shRNA GFP^+CD8^+ T_N and Control-shRNA GFP^+CD8^+ T_N (1×10^5/well, 96-well plate) were stimulated with anti-CD3Ab and anti-CD28 (2.5 μg/mL for each). Five days later, cells were recovered and analyzed with flow cyotmetry for measuring GFP^+CD8^+ T cells. (D) Unfractionated Ezh2-shRNA GFP^+CD8^+ T_N were stimulated with BABL/c mouse-derived DCs, or with IL-7 alone. Five days later, cells were recovered and analyzed using flow cytometry for measuring GFP^+CD8^+ T cells. Fold change of GFP^+CD8^+ T cells was calculated based on the output number of GFP^+CD8^+ T cells after culture divided by the input number of GFP^+CD8^+ T cells before culture. Data shown in B, C, and D are representative of 2 independent experiments.

that these cells could share some common properties. ESCs and NSCs characterized in Ramalho-Santos' et al. study are highly proliferating stem cells, whereas HSCs are quiescent cells [41]. In their study, ESCs were derived from the inner cell mass of the blastocyst stage of embryos, whereas NSCs were isolated from brain-derived neurospheres. Both ESCs and NSCs were highly purified after ex vivo cultures for gene expression profile analyses. In contrast, HSCs were freshly isolated from BM of normal B6 mice based on dual-dye efflux and HSC markers [41]. We found that many of these $CD8^+$ T_E-related stem cell genes were associated with cell cycle regulation, DNA replication and repair, and stress resistance. This was in agreement with our findings that a proportion of alloreactive $CD8^+$ T_E continually proliferated upon chronic exposure to alloantigens. Thus, genes controlling proliferation of ESCs and NSCs are an important component of the similarity between $CD8^+$ T_E and embryonic and neural stem cells.

However, $CD8^+$ T_E did not increase the expression of genes associated with pluripotency of ESCs. In contrast, they activated many other stem cell genes that are found to be important for controlling cell fate, differentiation, survival, self-renewal, and memory function in ESCs and NSCs, such as Uhrf1,Tacc3, Hells, Birc5, and Ezh2 [37,49-54,61]. For example, Ezh2 binds to chromatin and DNA during cell dividing, thereby preserving transcriptional programs and cell identity established during earlier response phase [62,63]. We found that $CD8^+$ T_E had the ability to sustain their effector functions over

numerous rounds of cell dividing upon chronic exposure to allogeneic stimuli. Previous reports have also suggested that $CD8^+$ T_E can become self-renewing memory T cells upon clearance of the target antigen [4-6,8,22]. These observations suggest that $CD8^+$ T_E share some common properties with ESCs and NSCs in the expression of stem cell transcriptional programs that are engaged in cell fate decision, self-renewal, survival, differentiation, and memory function.

Several lines of evidence suggest that Ezh2 may be important for antigen-driven T cell responses. We found that Ezh2 was abundantly expressed in antigen-activated $CD8^+$ T cells but not in $CD8^+$ T_N. Silencing Ezh2 inhibited $CD8^+$ T cell proliferation activated by TCR/CD28 costimulation and allogeneic DCs, which is consistent with a previous report of others [58]. Interestingly, knockdown of Ezh2 did not affect mature T cells to proliferate in response to homeostatic cytokine IL-7 alone. Thus, it is unlikely that inhibition of Ezh2 in alloreactive T_E can globally affect donor T cell immunity after allogeneic HSCT. However, further studies are necessary to investigate the impact of Ezh2 inhibition in antigen-activated T cell responses and GVHD.

Our results suggest that APCs may play an important role in regulating stem cell transcriptional programs in $CD8^+$ T cells. We found that alloreactive $CD8^+$ T_E continuously replicated in secondary allogeneic recipients and caused severe GVHD, but rapidly diminished in congenic recipients where alloantigens were absent. Thus, allogeneic stimuli rather than homeostatic factors are critical to the continuous replication in vivo of alloreactive $CD8^+$ T_E. This may explain why APCs are important for alloreactive T cell-mediated GVHD at both the induction and effector phase [13,23,64-66]. Other studies suggest that antigenic stimulation is also necessary for protective immunity during chronic infection [67-69]. It is likely that antigen stimulation sustains the replication of T_E through the activation of stem cell transcriptional programs. However, other nonantigenic stimuli, such as inflammatory cytokines and costimulatory signals, could also be important for regulating stem cell transcriptional programs in $CD8^+$ T_E. For example, $CD4^+$ T cells are found to be important for in vivo development of long-lasting $CD8^+$ memory T cells and are required for mediating chronic GVHD [70-72]. It is possible that signals derived from $CD4^+$ help T cells might affect the expression of these stem cell genes in antigen-activated $CD8^+$ T cells.

Stem cell transcriptional programs may also play an important role in alloreactive $CD8^+$ T_{MSC}. Gene expression profile analysis showed that these $CD8^+$ T_{MSC} were less differentiated as they did not produce cytotoxic molecules and inflammatory cytokines. A recent study suggests that activation of Wnt-signaling arrests the effector differentiation, whereas it enhances the generation of $CD8^+$ T_{MSC} with greater ability than mature memory T cells to proliferate and generate tumor-reactive T_E in vivo [21]. This further supports that $CD8^+$ T_{MSC} themselves do not mediate tissue injury, but can further differentiate into functional $CD8^+$ T_E [15]. Notably, 70% of stem cell genes activated in alloreactive $CD8^+$ T_{MSC} remained increased in $CD8^+$ T_E. It will be interesting to determine how these ESC- and NSC-related genes affect the proliferation and differentiation of $CD8^+$ T_{MSC}.

In summary, we have identified that "terminally differentiated" alloreactive $CD8^+$ T cells activate stem cell transcriptional programs that are normally expressed in ESCs and NSCs. This group of stem cell genes may play important roles in regulating the proliferation and persistence of alloreactive T cells upon chronic exposure to alloantigens in GVHD. Further exploring the specific roles of ESC- and NSC-related stem cell genes in chronically activated T cells could have significant impact on understanding and modulating pathogenic T cell responses in many other inflammatory conditions, such as chronic infections, autoimmune diseases, and rejection of grafted solid organs.

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